Performance of Two Tests for Measurement of Serum and Plasma Fructosamine in Dogs

M. Joan Massat[1]

Philip Johnson[2]

Charles E. Wiedmeyer[3]

[1]Boehringer Ingelheim Animal Health at Missouri Research Center,
[2]University of Missouri, College of Veterinary Medicine,
Department of Veterinary Surgery and Medicine,
[3]University of Missouri, College of Veterinary Medicine,
Department of Veterinary Pathobiology

Correspondence
M. Joan Massat, Missouri Research Center,
6498 Jade Road, Fulton, MO 65251.
E-mail: Joan.massat@boehringer-ingelheim.com

ABSTRACT

Objective

The goal of this study is to compare the performance of two assay methodologies for the measurement of fructosamine in canine serum and plasma. This study is not intended to validate either reagent, but to highlight differences between these reagents.

Background

Serum fructosamine is used clinically to examine average glucose level over a one to two week period. Determination of serum fructosamine can eliminate the necessity of multiple serum glucose measurements since it is not affected by short-term fluctuations in blood glucose levels. It is one of the most common alternatives used for assessment of glycemic control in diabetic patients.

Methods

A Nitroblue tetrazolium (NBT) fructosamine assay and a Fructosaminase™ fructosamine assay were tested for correlation, precision, stability, and interference for the measurement of fructosamine in canine serum and plasma.

Results

The NBT assay is more precise but low and intermediate fructosamine values were indistinguishable compared to the Fructosaminase™ assay. High levels of lipid, hemolysis, or icterus may interfere significantly with both methodologies.

Conclusions

Both assays are useful for measuring serum and plasma fructosamine levels in canine samples but they do not correlate well and cannot be used interchangeably. Storage conditions, matrix used, (serum or plasma), and interference from lipemia, icterus, and hemolysis affect these assays differently. This highlights the need to interpret patient results carefully, particularly when they may come from multiple laboratories or clinics.

INTRODUCTION

Fructosamine is a general term for ketoamines formed by non-enzymatic attach-

ment of glucose to proteins in the blood.[1] Fructosamine serum or plasma levels can be used to examine the average glucose level over a 1 to 2 week period since it is not affected by short-term fluctuations in blood glucose. Fructosamine is routinely used to assess glycemic control in diabetic patients. An advantage to measuring fructosamine over traditional measures of glycemic control, such as a blood glucose curve, is the need for a single, non-fasted plasma or serum sample rather than multiple samples. Glycated hemoglobin (HbA1c), commonly used in humans, is another test used to assess average glucose over a defined period of time but it is not commonly used in dogs because research has shown a large overlap between the levels of HbA1c in normal dogs and those with uncontrolled diabetes.[2] Diabetes is often diagnosed based on clinical signs such as polyuria, polydipsia, and glucosuria along with hyperglycemia, but these can occur for reasons other than diabetes, so fructosamine concentrations are a more reliable indicator of canine diabetes.[3]

Two types of assays are commercially available in the United States for automated fructosamine assays of human serum or plasma. These are the NBT assay and the Fructosaminase™ assay. No reagent for automated assays of fructosamine has been made specifically for veterinary use. Most research currently conducted on fructosamine measurements in the canine has been with NBT reagent which was validated for use in canine samples in in 1992.[3] Known drawbacks of this method are that it is affected by changes in ambient temperature and that substances other than a ketoamine (fructosamine) such as bilirubin and some vitamins can reduce the NBT and interfere with the test.[4] Our goal was to compare these two reagents on the same analyzer with the same samples and ascertain, if possible, which reagent is best for measurement of fructosamine in canine serum and plasma. To that end, correlation, precision, stability, and interference were measured in canine serum with the two reagents. In addition, serum, and plasma samples were compared

for both assays.

MATERIALS AND METHODS
Assays Used

Two assays using different methodologies for the measurement of serum and plasma fructosamine in dogs were examined; NBT reagent (Catachem Inc., Oxford, CT), and the Fructosaminase™ assay (Diazyme Laboratories, Poway, CA). The NBT reagent depends on ketoamines to reduce NBT in alkaline conditions to form a purple formazan complex. It is a colorimetric kinetic assay where the rate of product formation is measured.[1] The Fructosaminase™ reagent first digests glycosylated serum proteins into low molecular weight fragments and then uses an amadoriase enzyme called Fructosaminase™ to oxidate the products. This yields peroxide which undergoes a trinder reaction where H_2O_2 is reduced to H_2O and a colored compound is produced in proportion to the amount of H_2O_2 present.[5] This reagent is described as a colorimetric endpoint reaction. Changes in light absorbance is measured to quantitate fructosamine for both reagents. Because these reagents utilize different chemical reactions to quantitate fructosamine values, interfering substances, stability, and precision differ.

The fructosamine assays were utilized on an AU480e automated clinical chemistry analyzer (Beckman Coulter, Brea CA). Reagent for LIH (lipemia, icterus, and hemolysis (Beckman Coulter, Brea CA) was utilized on the same automated chemistry platform. LIH is a semi-quantitative test intended to measure interference in human plasma and serum.[6]

Samples

Unfiltered beagle serum (Lampire Biologicals, Pipersville, PA) served as a control for interference testing. This serum was drawn from normal healthy dogs that were fasted before collection. The serum was shipped on dry ice and subsequently stored at -20ºC until analysis.

All samples used for precision, stability, and serum vs. plasma, were obtained fresh

from healthy non-fasted beagles at Missouri Research Center. These samples were obtained with a conditioning animal procurement statement for reference interval data, but sufficient sample existed to use them for this study as well. Missouri Research Center is AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care International) accredited. All of these samples were analyzed on the day of collection. Canine plasma was obtained from whole blood placed in lithium heparin tubes and collected following centrifugation at 1900g for 20 minutes. For Serum samples, blood placed in tubes with no anticoagulant was allowed to clot for approximately 30 minutes at room temperature and centrifuged at 1,900g for 20 minutes and the serum collected. Both stored and fresh samples were used for correlation. Fresh samples from diabetic canines were not available. Serum from diabetic dogs with various states of glycemic control were refrigerated for short-term storage or frozen, and then shipped on cold-packs and stored at -20ºC until analysis. The samples varied from a few days old to approximately three weeks old and most had undergone 1-2 freeze thaw-cycles prior to analysis. These samples were obtained from local veterinary clinics and from an IDEXX reference laboratory (samples were serum left over after diagnostic testing was completed for other purposes). Controls were run with both reagents with each set of study samples included in this study.

Assay Performance

Correlation

To assess correlation, sixty-eight fresh canine serum samples from Missouri Research Center and one hundred stored serum samples were analyzed by both methods. Sixty-nine of the stored serum samples were from the canine serum collected at Missouri Research Center and stored at -70 ºC prior to analysis. Thirty-one of the stored samples were from diabetic animals, which were shipped to Missouri research center.

Precision

Canine serum samples of relatively low

(n=5), intermediate (n=7), and high (n=4) fructosamine levels as determined via a survey of samples used for correlation data were selected to investigate precision with pooled samples. Fructosamine results obtained with both NBT and Fructosaminase™ assays were assessed to determine which samples should be used for each pool. For intraday precision, ten runs were done at each level with each assay method. For inter-day precision, each level was run once a day for 10 days with each assay method. Samples were stored in a freezer capable of a nominal temperature of -20ºC between each test.

Stability

To assess stability of fructosamine levels for each method, fructosamine was measured for 64 fresh serum samples and results were compared after 1 month of storage at -70ºC and -20ºC for both tests. Each sample was measured once before and after storage. Storage at 4ºC and 25ºC was not done in this study. A study by Jensen in 1992 showed that canine serum fructosamine is stable for 5 days at 4ºC or 25ºC as measured by the NBT method.[3]

Serum vs. Plasma

Both assays list serum as the intended matrix. To examine the utility of these tests in plasma, paired plasma/serum samples (n=79) were analyzed by the NBT and the Fructosaminase™ assay.

Interference

The effects of hemolysis, icterus, and lipemia on fructosamine measurement with both assays was examined via the addition of interfering substrates to serum samples. The control serum that was used had baseline levels of approximately 40-99 mg/dL lipid and 50-99 mg/dL hemolysis.[6] Levels of interfering substances added were: 62-2000 mg/dL of lipid, 12.5-200 mg/dL of hemoglobin (Hgb) and 0.625-20 mg/dL bilirubin.

A. Lipemia

Intralipid® 20% emulsion (Sigma Aldrich, Saint-Louis, MO) was used to create lipemic samples as done by

Steen et al.[7] Control serum was spiked with varying amounts of Intralipid to create levels of 62.0, 120, 250, 500, 1,000, and 2,000 mg/dL in serum. Two aliquots were made for each concentration and each aliquot was run with both methods. The average for each method was used for calculations. A blank for each level was created (control serum diluted with saline) and run for each method.

B. Icterus

Conjugated bilirubin is more soluble and unconjugated bilirubin binds to albumin.[8] Therefore, both types of bilirubin were tested. Unconjugated bilirubin (Sigma-Aldrich, St. Louis, MO) and bilirubin conjugate (VWR, Radnor, PA) were obtained. Serum bilirubin standards including blanks were created by procedure used by Gupta and Stockham.[9] Levels of 0.625, 1.25, 2.50, 5.00, 10.0 and 20.0 mg/d bilirubin were created for both unconjugated and conjugated bilirubin. Two aliquots were made for each concentration and each aliquot was run with both methods. The average for each method was used for calculations. Blanks were also run with both methods.

C. Hemolysis

A hemolytic solution was created using the procedure described in Hillstrom et al.[10] Initial Hgb concentration was measured on a Siemens Advia 120 hematology instrument (Siemens, Munich Germany) and serial dilution was used to create samples with levels of 12.5, 25.0, 50.0, 100, and 200 mg/dL HGB. Control serum was spiked with saline and diluted in the same manner as the hemolyzed spiked serum to create blanks

for each level. One aliquot of each level was made for testing hemolysis. Samples and blanks were analyzed using both fructosamine assays.

In addition, LIH tests were performed on all samples from diabetic canines.

Statistics and Calculations

All statistics and calculations were done using Microsoft Excel 2010 except the Spearman's Rank Correlation, which was done with SAS version 9.4. Analyze-it® method-validation edition for Excel was used to create the Bland-Altman plot.

Paired two-tailed T tests for means with α=0.05 were done to examine statistical significance of the difference between serum and plasma fructosamine and the differences between fresh and stored samples.

For pooled precision, the mean, standard deviation, and CV were determined for each fructosamine level, and then the average CV was computed for each method.

The percent difference between the average fructosamine result for each reagent and the blank standard average result at each level of interference was calculated to ensure that differences in concentration were not due to dilution of the samples. For interference testing %Difference=Absolute value (spiked average-blank average)/blank

Figure 1: *Fructosaminase™ vs. NBT Bland-Altman plot This Bland-Altman plot highlights systemic and proportional error between measurements with the two reagents. The solid line represents the mean of the difference between the two measurements which is also called the bias. The dotted lines show the limits of agreement (Mean +/- 1.96s). The plot reveals that error increases for higher fructosamine values.*
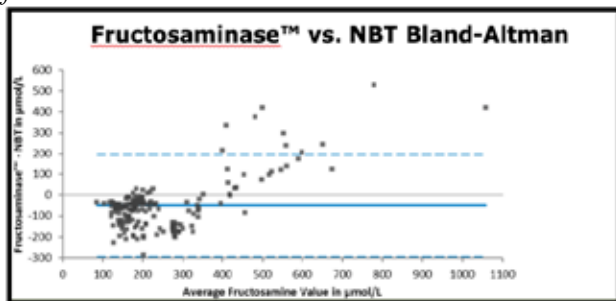
Table 1. *CV of Mean Fructosamine Values*

Reagent	NBT	Fructosaminase™
Pooled Precision		
Intraday Low	0.8%	2.9%
Intraday Medium	0.4%	0.6%
Intraday high	0.6%	2.2%
Interday Low	18.3%*	23.4%
Interday Medium	14.4%*	2.1%
Interday High	8.5%*	4.5%

Some replicates could not be used due to reaction errors

average*100%.

To examine correlation, the data was first assessed visually for normality with a histogram to determine whether to use parametric or nonparametric statistics. Since the data were not normally distributed, Spearman's correlation coefficient was calculated. A Bland-Altman plot was also created. The Bland-Altman plot was created by plotting the difference in the assays (Fructosaminase™-NBT result) on the y axis and the average value of the two assays on the x axis.

RESULTS

Correlation

The Spearmen's Rank Correlation test showed that the methods did correlate (p<0.0001). The Spearman's correlation coefficient, rs, was 0.65012. The Bland-Altman Plot reveals that error appears to increase for higher values of fructosamine. (Figure 1).

Precision

Both assays revealed acceptable intraday precision (i.e. <10%). (Table 1) The interday precision was high (23.4%) for the low levels with the Fructosaminase™ assay but was <5% for medium and high levels of fructosamine. The interday precision was uncertain for the NBT reagent because reaction errors occurred during analysis (out of ten replicates, one error, two errors, and four errors occurred at the low, medium and high levels, respectively).

Stability

Storage of samples for 1 month at -20ºC yielded a statistically significant decrease (p<0.0001 at α=0.05) (230 μmol/L for fresh samples to 208 μmol/L after storage) in fructosamine values when analyzed with NBT reagent. The Fructosaminase™ reagent showed no statistically significant difference (p=0.1878 at α=0.05) after storage of serum samples for one month at -20ºC (106 μmol/L for fresh samples to 109 μmol/L after storage). After 1 month of storage at -70ºC the average fructosamine value for samples for both assays showed a statistically significant decrease; NBT went from 230 μmol/L down to 194 μmol/L and Fructosaminase™ values went from 102 μmol/L down to 86 μmol/L (both p<0.0001 at α=0.05). Although 64 samples were assessed for 1 month stability at -20ºC and -70ºC with both reagents only 33 samples could be used for statistics to assess the Fructosaminase™ reagent. This was because of control failure at the 1-month time-point for some of the samples collected. Samples were not re-assayed as they needed to be assayed at the same time-point for both reagents. The lower number of samples stored at -70ºC could have made statistical differences in stability more difficult to detect, but since statistical signficiance was shown for both reagents this was not the case.

Serum vs. Plasma

The investigation of the difference in fructosamine results for serum vs. plasma in healthy beagles yielded a statistically significant difference p<0.0001 with both reagents at α=0.05. With NBT reagent the average serum fructosamine concentration was 209 μmol/L with a CV of 7.1% and the average plasma was 224 μmol/L with a CV of 8.4%. With the Fructosaminase™ reagent the average serum fructosamine concentration was 124 μmol/L with a CV of 28.0% while the average plasma was 108 μmol/L with a CV of 24.4%. Seventy-nine samples were run for serum vs. plasma, but only 42 of these could be used for statistical analysis of NBT reagent as the NBT control failed on one of the sample collection days. The

Table 2. *Percent of Serum Samples from Diabetic Canines Showing Interference*

	No Interference	1+	2+	3+	4+	5+
Lipemia	None	None	22.6	9.7	38.7	29.0
Hemolysis	22.6	25.9	22.6	29.0	None	None
Icterus	77.4	22.6	None	None	None	None

samples were not re-assayed as this was a comparison of fresh samples. It is harder to show statistical significance for a smaller number of samples but since a statistically significance difference was shown between serum and plasma for both reagents it is clear that the smaller number of samples for the NBT method did not affect results.
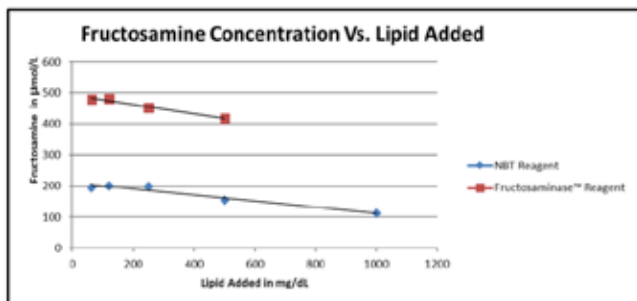
Interference

Significant interference with both reagents was observed. The percent difference between the spiked samples and blank samples showed that changes in fructosamine values were not due to dilution effects. For hemoglobin, unconjugated and conjugated bilirubin, there was a greater difference in fructosamine values between the spiked and blank samples as assayed with the NBT reagent. For example, at 200 mg/dL HGB there was a 33% difference for NBT and a 7.6% difference for the Fructosaminase™ reagent. For 20 mg/dL conjugated bilirubin there was a 44% difference with NBT and a 22% difference with the Fructosaminase™ reagent. For 20 mg/dL unconjugated bilirubin the difference was 111% for NBT and 13.6% for Fructosaminase™ reagent. Percent difference at 500 mg/dL lipid was 20.0% for NBT and 19.3% for Fructosaminase™ reagent. At lower levels of lipid the % difference was higher with Fructosaminase™ reagent than with NBT reagent. For the full data table see supplementary data.

Increasing levels of lipemia resulted in a decrease in measured fructosamine for both reagents (Figure 2). Icterus from both unconjugated and conjugated bilirubin decreased fructosamine concentrations using the Fructosaminase™ reagent but increased the fructosamine value using NBT reagent. There was less interference from bilirubin for the Fructosaminase™ reagent (Figure 3). Added hemoglobin decreased measured fructosamine with the Fructosaminase™ reagent but increased it with the NBT reagent (Figure 4).

In addition, all patient samples from diabetic canines showed some level of interfering substances and most showed more than one type of interference. (Table 2).

Figure 2-*Fructosamine Concentration Measured Vs. Lipid Added*

62-2000 mg/dL of lipid was added to control canine serum. Linear regressions show the effect of increasing levels of lipid in canine serum on the measurement of fructosamine by NBT (R2=0.9534) and Fructosaminase™ (R2=0.969) reagents. Concentrations of fructosamine measured with both reagents decreased when the concentration of lipid increased. Measurements with Fructosaminase™ reagent at higher concentrations of lipid gave only instrument errors and no numeric results. For reference, 22.6% of samples from diabetic animals showed lipemia of 2+ (100-199 mg/dL), 9.7% showed a level of 3+ (200-299 mg/dL), 38.7% showed a level of 4+ (300-500 mg/dL) and 29.0% showed a level of 5+ (>500 mg/dL lipid).

Fructosamine Concentration Vs. Lipid Added

DISCUSSION

Fructosamine concentrations have been shown to be a reliable indicator of canine diabetes and a useful indicator of glycemic control in dogs.[11] These assays work by different mechanisms. It is important to highlight the differences between these two assays when used for canine samples because these two reagents do not correlate very well for fructosamine measurement in canine serum (Figure 1). The difference is sufficient that diagnosis could be incorrect if these reagents are used interchangeably. Fresh samples from healthy beagles (n=68) ranged from 188.7 to 381.8 umol/L when assayed with the NBT method and 14.5 to 224.3 umol/L when assayed with the Fructosaminase™ method. The animal with the level of 14.5 had a level of 242.2 with the NBT test. It is unknown why this sample had very low results with the Fructosaminase™ assay. Controls passed before and after samples were run on the day of analysis and no instrument errors occurred. It is possible that this sample contained high levels of lipid, which reduced the fructosamine result measured with both methods. The sample was not re-assayed because these tests were initially run for the purpose of deciding which fructosamine test to use for another study. No interference data was collected at that time. When it became apparent that significant discrepancies between the results of the two tests existed a protocol was written to collect data on interference.

One limitation of this study is that fresh samples from diabetic canines were not available. It would have been useful to use these samples to explore correlation and stability. We used paired T-tests to compare the reagents so each sample was compared to the exact same sample which would have undergone the same storage conditions. There is still the possibility that storage conditions may affect the results of these tests differently in the same sample. However, looking at correlation of samples collected under a variety of conditions is useful because differences in sample collection or analysis can occur. For many smaller veterinary clinics, particularly in rural or isolated areas, there may be differences in sample collection and storage. While ASVCP recommendations exist, regulations such as CLIA do not exist for veterinary samples in the United States. Some clinics may refrigerate or freeze samples to be run at a later date, while others may ship samples to a reference laboratory. Reference laboratories follow strict protocols, but different reference labs may use different test methodologies and have different reference intervals. This may cause much confusion when examining patient histories. Because fructosamines are proteins with non-enzymatically attached glucose molecules, it is not expected that short-term storage at refrigerated or frozen conditions would degrade the analyte. However, alteration of other substances in the samples, such as interfering substances, may cause inaccurate measurement of fructosamine.

Assessment of interference is important for evaluating any diagnostic or survey biochemical assay because of the clinical decisions dependent on their outcome. Assay manufacturers commonly list interference studies done in human serum on package inserts. According to the package insert for the Fructosaminase™ reagent "less than 10% deviation" occurs for unconjugated bilirubin up to 7.5 mg/dl, conjugated bilirubin up to 5 mg/dL, triglyceride concentrations up to 2000 mg/dL, and hemoglobin concentrations up to 200 mg/dL.[5]

Catachem states that for their NBT reagent "no significant effect on the accuracy of the procedure" occurs for less than 100 mg/dL of hemoglobin, less than 1,200 mg/dL triglycerides, and less than 4 mg/dL unconjugated bilirubin when tested in human serum.[1] No data on interference from conjugated bilirubin was on the Catachem package insert. No data on interference in canine serum was available on the package inserts for either reagent and no direct comparison of interference between the two different assays was available. Assess-

Figure 3-*Fructosamine Concentration vs. Bilirubin Added*
0.625-20.0 mg/dL of conjugated and unconjugated bilirubin was added to control canine serum. Regression analysis shows the effects of increasing levels of conjugated and unconjugated bilirubin in canine serum on the measurement of fructosamine with each reagent (Regressions are linear with the exception of unconjugated bilirubin with Fructosaminase™ reagent, which is polynomial). Both conjugated ($R^2=0.9953$) and unconjugated bilirubin ($R^2=0.9566$) produced falsely increased Fructosamine concentrations with NBT reagent whereas measurement with the Fructosaminase™ reagent caused both conjugated ($R^2=0.9917$) and unconjugated bilirubin ($R^2=0.9997$) to produce falsely low levels of fructosamine although the effect leveled off around 13 mg/dL bilirubin added. For reference, 22.6% of the samples from diabetic animals we tested had a 1+ icterus (2.5-4.9 mg/dL bilirubin).
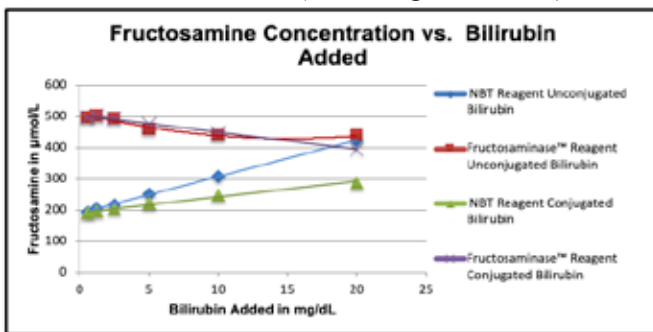

Figure 4-*Fructosamine Concentration vs. HGB Added*
12.5-200 mg/dL of Hgb was added to control canine serum. Linear regression shows the effect of increasing levels of Hgb in canine serum on the measurement of fructosamine by NBT reagent ($R^2=0.9952$). Polynomial regression shows the effect of increasing levels of Hgb in canine serum on the measurement of fructosamine by Fructosaminase™ reagent ($R^2=0.9669$). Addition of Hgb artificially increased the concentration of fructosamine in samples when measured with NBT reagent. Addition of Hgb up to 100 mg/dL did not cause significant change in the fructosamine concentration but Hgb levels above 100 mg/dL artificially decreased the fructosamine levels. For reference, 25.9% of the serum samples from diabetic canines that we tested had a 1+ hemolysis (50-99 mg/dL Hgb), 22.6% had a 2+ (100-199 mg/dL Hgb) and 29.0 had a 3+ (200-299 mg/dL Hgb).
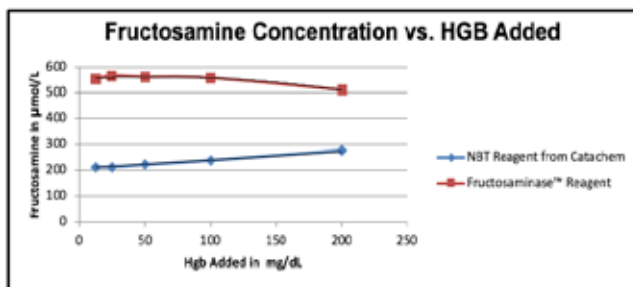
ment of interference in veterinary samples is particularly important for small animals because lipemic and hemolyzed samples occur frequently and icteric samples also occur, although less frequently.

The differences between the methods as shown in the Bland-Altman plot (Figure 1) highlight the need to consider the effects of interference on assay results. Note that there is a larger difference between the two methods at higher levels of Fructosamine. This may be due to increased concentrations of interfering substances. We showed that increasing levels of substance interference

in the spiked serum samples affected the results of both assays but in different manners. Lipemia resulted in a decrease in measured fructosamine for both reagents (Figure 2). Interference with bilirubin (Figure 3) and hemoglobin (Figure 4) decreased the fructosamine results using the Fructosaminase™ assay (apart from a slight increase between 12.5 and 25.0 mg/dL of icterus for the Fructosaminase™ assay). These interferences resulted in increased fructosamine levels using NBT assay. The differences in measured fructosamine levels may affect clinical decisions because the same dog may appear to have very different levels of fructosamine depending on the interference in the sample and the type of assay that was used. This means that it is very important to make sure that the reference interval that is used was created with the same reagent used to assay the patient's fructosamine levels. In addition, patient records should include reference interval data or state what type of assay was used to determine fructosamine levels.

In addition to spiking control plasma with interfering substances to show that they could cause measurement differences we measured lipemia, icterus, and hemolysis in 31 samples obtained from diabetic canines. All of the samples showed at least a 2+ level of lipemia and 77.4% of the samples had at least a 1+ hemolysis (See Table 2 for explanation of levels). Although only 22.6% of the samples we tested showed icterus, this parameter is still of importance for animals with comorbidities. Our findings for these samples are expected as hyperlipidemia and high cholesterol are common findings in diabetic dogs, especially those with poor glycemic control.[12]

In dogs, serum triglyceride levels of 150-400 mg/dL are considered mildly elevated, while levels of 400-1000 mg/dL are considered intermediately to markedly elevated.[12] Cholesterol is another lipid found in serum that may cause additional interference, particularly in diabetic canines. Cholesterol values of 500-750 mg/dL are considered moderately increased in diabetic dogs.[12] For hemolysis, levels of 20-100 mg/dL of hemoglobin are considered slightly hemolyzed, 100-300 mg/dL moderate and greater than 300 mg/dl, marked.[13] Hemolysis may also occur frequently due to poor venipuncture technique or lipemia in the sample.[13] Icterus is a less common finding in diabetic dogs but can occur in dogs with hemolytic anemia or secondary to cholestatic liver disorders where bile flow is obstructed or diminished.[13] Total bilirubin in dogs with bile duct obstruction may be up to 30 mg/dL.[13]

Because multiple types of interference may occur in the same sample it is difficult to assess the overall effects of interfering substances on fructosamine measurement. For example, if we take a sample with 4+ lipemia, 2+ hemolysis and 1+ icterus run with the NBT reagent we may see a 20% reduction of fructosamine measured due to lipemia, a 15% increase in fructosamine level due hemolysis, and a 5% increase in fructosamine level due to icterus. That would mean the level of fructosamine measured was unchanged. However, if the same sample was measured with the Fructosaminase™ reagent the hemolysis would not be likely to significantly affect the fructosamine measured but the lipemia might decrease the concentration measured by 15%, and the icterus might further decrease it by 5%.

One limitation of this study is that we assumed that each interfering substance affects measurement independently. It is possible, however, that each type of interference does not affect the measurement independently. For example, perhaps lipemia decreases with one test when it is the only interference present and hemolysis increases the result when the only interference present is hemolysis, but when combined these interfering substances increase the concentration measured or perhaps have no effect at all. It would be very difficult to take into account all the possibilities.

Both fructosamine assays have advantages and may be useful in assessing glyce-

mic control in diabetic dogs. The NBT assay has better intraday precision, but reaction errors occurred with some samples during interday precision. The analyzer detected abnormal reaction chemistry and flagged some of the results. The exact cause of these errors is not known. These interday precision runs were not re-assayed because that would subject the pooled plasma for one test to a different number of freeze-thaw cycles. The Fructosaminase™ reagent did not have acceptable inter-day precision at low levels (for low levels we considered <20% CV acceptable and for medium and high levels we considered <15% CV acceptable). Inter-day precision was acceptable for medium and high levels <5% CV were obtained. Significant interference from lipemia, icterus, and hemolysis was observed for both reagents, although interference affected the assays in different ways.

Analysis of canine serum with a glycated albumin assay might also have been useful to show which fructosamine reagent was more accurate since there is no gold-standard for assessing fructosamine in canines. Glycated albumin has been shown to correlate to fructosamine in canine serum when glycated albumin was measured with a Lucica GA-L assay kit and fructosamine was measured with an FOD TOOS method (a different enzymatic method than the Fructosaminase™ method we used).[14] The Lucica-GA-L is a reagent kit for automated analysis of human serum, which is sold in Japan but was not available in the United States, so this assay was not used for comparison. Fructosamine results should correlate well to glycated albumin assays as glycated albumin is the most common glycated protein in fructosamine. However, available methods in the United States were not standardized for use in dogs and are too labor intense.

Studies show that affinity chromatography can underestimate the amount of glycation because molecules with only one glycated site may not be able to bind tightly to the column.[15] An HPLC method has been developed to assay glycated albumin in human samples, but it is labor intense and requires both an anion exchange column to separate albumin from other proteins and a boronate affinity column to separate glycated and non-glycated albumin.[16] This method is not practical for small veterinary laboratories due to the expense of the equipment and the expertise required. Canine glycated albumin would need to be special ordered to calibrate for such a test as well. Furthermore, it is more efficient to run fructosamine samples on a clinical chemistry analyzer as multiple analytes can be easily run on the same machine with the same sample.

When the tests were compared in different storage conditions, storage at -20ºC for 1 month resulted in a statistically significant decrease in serum frucostamine levels as measured with the NBT reagent, but not the Fructosaminase™ assay. With the NBT assay a 2.8% difference occurred, which is not clinically relevant. It is important to note here that statistical difference does not always mean that a clinically relevant difference exists.

Samples assayed with NBT reagent stored at -20ºC showed a 9.6% difference, which may not be clinically relevant in some cases. NBT reagent and Fructosaminase reagent both showed a 15.7% difference at -70ºC, which may be clinically relevant depending on how high the patient's fructosamine are. At -70ºC, both tests showed a statistically significant decrease in fructosamine values. Although the differences in fructosamine when stored at these conditions for 1 month may not be enough to be clinically relevant, care should be taken for long-term storage of samples. The decrease in fructosamine values was higher after storage at -70ºC for 1 month compared to -20ºC. Therefore, -20ºC is preferable for long-term storage of samples. The fructosamine itself may be very stable at many storage conditions, but because interfering substances may be affected by different storage conditions, stability should not be assumed, and care should be taken when storing samples.

It would have been useful to assess short-term storage of samples at -4ºC as well, but that was not done in this study.

Finally, both serum and plasma can be used to assay for fructosamine in dogs, but it is important to remember that results differ slightly depending on whether serum or plasma is used. A sample-specific and assay-specific reference interval should be used while interpreting fructosamine results. Furthermore, it may be a good idea to consider a breed-specific reference interval. The domestic dog is a very diverse species and more emphasis should be placed on using the most specific reference intervals available.[17] Samples from healthy animals for the purposes of this study were all from beagles fed a laboratory diet. While this study served the purpose of highlighting differences between the reagents, lack of diversity in the animals used is an important limitation of this work.

ACKNOWLEDGEMENTS

REFERENCES

1. DiscretPak™ Fructosamine for the Olympus AU Series [Product Use Guide]. Catachem Inc., Oxford, *CT Rev*: BA92015.

2. Delack JB, Stogdale L. Glycosylated Hemoglobin Measurement in Dogs and Cats: Implications for its Utility in Diabetic Monitoring. *Can Vet J*. 1983;24:308-311.

3. Jensen AL. Serum fructosamine in canine diabetes mellitus. An initial study. *Vet Res Commun*. 1992;16(1):1-9. doi:10.1007/bf01839201.

4. Danese E, Montagnana M, Nouvenne A, Lippi G. Advantages and pitfalls of fructosamine and glycated albumin in the diagnosis and treatment of diabetes. *J Diabetes Sci Technol*. ;9(2):169–176. doi:10.1177/1932296814567227

5. Glycated Serum Protein Assay [Package Insert]. Diazyme Laboratories Poway, CA; June 2011.

6. LIH [Product Use Guide], Beckman Coulter, Brea, CA

7. Steen G, Klerk A, Laan KVD, Eppens EF. Evaluation of the interference due to haemoglobin, bilirubin and lipids on Immulite 2500 assays: a practical approach. *Ann Clin Biochem*. 2011;48(2):170-175. doi:10.1258/acb.2010.010187.

8. Leib M. Central Veterinary Conference. In: Icterus in Dogs and Cats: A Practical Diagnostic Approach.

9. Gupta A, Stockham SL. Negative interference of icteric serum on a bichromatic biuret total protein assay. *Vet Clin Pathol*. 2014;43(3):422-427. doi:10.1111/vcp.12154.

10. Hillström A, Hagman R, Tvedten H, Kjelgaard-Hansen M. Validation of a commercially available automated canine-specific immunoturbidimetric method for measuring canine C-reactive protein. *Vet Clin Pathol*. 2014;43(2):235-243. doi:10.1111/vcp.12150.

11. Jensen AL, Aaes H. Reference interval and critical difference for canine serum fructosamine concentration. *Vet Res Commun*. 1992;16(5):317-325. doi:10.1007/bf01839181

12. Johnson MC. Hyperlipidemia Disorders in Dogs. Compendium on Continuing Education for the Practicing Veterinarian-North America Edition. 2005;27(5):361-370

13. A Resource for Veterinary Clinical Pathology. *eClinpath*. http://www.eclinpath.com/

14. Sako T, Mori A, Lee P, et al. Diagnostic Significance of Serum Glycated Albumin in Diabetic Dogs. *J of Vet Diagn Invest*. 2008;20(5):634-638. doi:10.1177/104063870802000517

15. Vorberg E. HPLC assay for serum glycated albumin. *Diabetologia*. 1992;35(7):699-700. doi:10.1007/bf00400267

16. Shima K, Ito N, Abe F, et al. High-performance liquid chromatographic assay of serum glycated albumin. *Diabetologia*. 1988;31(8):627-631. doi:10.1007/bf00264772.

17. Lawrence J, Chang YM, Szladovits B, Davison LJ, Garden OA. Breed-specific hematological phenotypes in the dog: a natural resource for the genetic dissection of hematological parameters in a mammalian species. *PLoS One*. 2013;8(11):e81288. Published 2013 Nov 25. doi:10.1371/journal.pone.0081288