Statement in veterinary textbooks regarding the effects of bilirubin on the refractometric total protein concentration are conflicting; some textbooks state that hyperbilirubinemia (icterus) has no effect, whereas others list hyperbilirubinemia as a cause of a false increase in refractometric total protein concentrations. Human clinical biochemistry textbooks also state that hyperbilirubinemia will cause a false increase. Published data indicate that hyperbilirubinemia has little or no effect, but the bilirubin concentrations in those studies are either not provided or represent only minor increases from physiologic concentrations (<1.0 mg/dL).

Establishing whether bilirubin affects refractometry will allow veterinarians to properly interpret total protein concentrations when there are differences between refractometric total protein and biuret assay-derived total protein concentrations in icteric sera. Recently, pseudohypoproteinemia was reported in a hyperbilirubinemic dog. The refractometer measurement was in the reference interval, whereas the biuret assay total protein concentration was decreased. The lower biuret assay total protein concentration was attributed to interference caused by bilirubin and biliverdin, but it was not reported whether the refractometric method was affected by the hyperbilirubinemia.

Refractometers measure the refractive index (n), or the bending of light when it passes through a liquid. Refraction (r), also known as refractive index difference, is the difference between the refractive index of an aqueous solution and water (ie, r = n – 1.333); the refractive index of water is 1.333. A refractive index is dependent on the soluble solids in the liquid, and both the TS% and the type of soluble particles in a solution alter the refractive index. Suspended particles do not alter the refractive index.

When the liquid is plasma or serum, the refractive index is proportional to the TS% (g of solute/100 g of solution × 100%). The refractometric estimation of total protein concentration includes the assumption that changes in the refractive index are caused by changes in total protein concentration, and the concentrations of other soluble solids have not changed. However, higher concentrations of soluble solids such as glucose, urea, electrolytes, and lipoproteins will result in a higher TS% and thus a higher refractive index. Considering the contribution of bilirubin to the TS%, even the amount of bilirubin in extremely icteric sera should not interfere with the refractometric total protein concentration.
Handheld refractometers are common in many veterinary laboratories and clinics and provide quick and inexpensive estimations of total protein concentrations in serum, plasma, or other body fluids. The total protein scale of clinical refractometers is typically calibrated for the normal soluble constituents of human plasma. Various equations or relationships have been used to convert refractive indices of human serum or plasma to total protein concentrations. It is also reported that calibration factors for converting refractive indices to total protein concentrations are different among species. On the basis of available information, different total protein concentrations may be found when different methods are used to convert a refractive index to a total protein concentration. However, observed differences among refractometers should not be influenced by the presence of increased bilirubin concentrations.

The purpose of the study reported here was to confirm or refute the supposition that hyperbilirubinemia causes falsely increased refractometric serum total protein concentrations. Because a variety of refractometers are available for a clinical laboratory, 3 refractometers were used and the refractometric data were compared with data from a biuret assay–based automated spectrophotometric method.

Materials and Methods

Blood collection—Venous blood samples were collected with syringes from 2 healthy Greyhounds from which food had been withheld overnight and then transferred to evacuated tubes. After approximately 30 minutes, tubes were centrifuged and nonlipemic and nonhemolyzed sera were harvested.

Bilirubin stock solution and preparation of icteric sera—Bilirubin powder (40 mg) was dissolved in 10 mL of 0.1M NaOH to prepare a stock bilirubin solution of nearly 400 mg/dL. Diluted stock solutions were mixed with a constant volume of sera (1:9 bilirubin solution mixed with serum) to create bilirubin concentrations near 1, 2, 4, 6, 8, 10, 15, 20, 25, 30, 35, and 40 mg/dL. The 0.1M NaOH solution was mixed with serum in the same ratio.

Refractometric total protein concentration measurements—Three temperature-compensated refractometers were used: a refractometer (refractometer 1) with total protein concentration (g/dL) and refraction scales, a refractometer (refractometer 2) with a TS% scale, and a refractometer (refractometer 3) with a total protein concentration scale (g/dL). Each refractometer was calibrated with deionized water to 1.000 on its urine specific gravity scale prior to analyzing samples. Because a conversion table for refractometer 2 was not found in the literature, its TS% values were first converted to refraction values (r) with the aid of a conversion table reported by Wolf and then to total protein concentrations in 4 ways: the conversion table, a conversion table for refractometer 1, a canine conversion formula, and a multispecies conversion formula.

To assess the analytic precision of the refractometric methods, 2 investigators (AG and a medical technologist) determined refractometric values in the unspiked serum samples and the mean of those values was calculated. This process was performed 8 times for unspiked sera of 2 dogs.

For the assessment of bias, the refractometric values of each unspiked and spiked sample were determined by the same 2 investigators with each refractometer. The mean values of the 2 readings were recorded for data analysis.

Clinical biochemical assays for serum total protein, total bilirubin, and direct bilirubin concentrations—Clinical biochemical assays were completed on the day of blood collection with an automated analyzer and the manufacturer’s reagents. The total protein assay (2-point-end, bichromatic biuret assay) was used to measure total protein concentrations in the unspiked serum samples. During the previous 5 months and including the weeks of this study, the CV for the biuret assay was 1.5% for commercial level 1 control serum (mean ± SD concentration, 6.0 ± 0.1 g/dL) and 1.5% for commercial level 2 control serum (mean concentration, 4.4 ± 0.1 g/dL). Unspiked sera of both dogs were assayed 8 times, and the mean concentration for each dog was calculated.

The total bilirubin assay was a modification of the Malloy-Evelyn method and the direct bilirubin assay was based on the Jendrassik-Grof procedure; automatic dilutions were completed for samples with bilirubin concentrations > 35 mg/dL.

Statistical analysis—The CV (%) was calculated for the repeated refractometric and spectrophotometric measurements of total protein concentrations in sera from both dogs. Difference plots were created to assess agreement among the refractometric total protein concentrations.

Results

Analytic precision of refractometric and spectrophotometric total protein assay methods—For the unspiked canine sera of the 2 dogs, the mean refractometric total protein concentrations were 6.5 g/dL (CV, 0.0%) and 6.2 g/dL (CV, 0.0%) for refractometer 1, 6.2 g/dL. The CV (%) was calculated for data analysis.

Table 1—Refractometric total protein concentrations in unspiked sera of 2 dogs. Difference plots were created to assess agreement among the refractometric total protein concentrations.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Dog 1</th>
<th>Dog 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSI% (g/100 g × 100%) from refractometer 2</td>
<td>7.6</td>
<td>7.3</td>
</tr>
<tr>
<td>Refraction (conversion table from Wolf)</td>
<td>0.0139</td>
<td>0.0133</td>
</tr>
<tr>
<td>Total protein concentration</td>
<td>6.2</td>
<td>5.8</td>
</tr>
<tr>
<td>Conversion table from Wolf (g/dL)</td>
<td>6.3</td>
<td>6.0</td>
</tr>
<tr>
<td>Conversion table for refractometer 1 with a total protein concentration (g/dL)</td>
<td>6.2</td>
<td>5.8</td>
</tr>
<tr>
<td>and a refraction scale (g/dL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Canine formula (g/dL)</td>
<td>5.8</td>
<td>5.5</td>
</tr>
<tr>
<td>Multispecies formula (g/dL)</td>
<td>6.3</td>
<td>6.0</td>
</tr>
<tr>
<td>Biuret assay total protein concentration (g/dL)</td>
<td>6.2</td>
<td>5.8</td>
</tr>
</tbody>
</table>

*Formula for canine sera: total protein concentration = (430 × r) – 0.20, where r is refraction. †Formula derived for the combination of bovine, canine, and equine sera: total protein concentration = (482 × r) – 0.41.
The mean spectrophotometric total protein concentrations in the unspiked sera were 6.2 g/dL (CV, 1.4%) and 5.8 g/dL (CV, 1.4%) for the 2 dogs, respectively. The CV percentages were consistent with the laboratory's daily quality control data for level 1 and level 2 control sera.

Agreement among refractometric total protein concentrations—For refractometer 2, different total protein concentrations were derived by different methods (Table 1). Because the refractometer 2 total protein concentrations determined with the conversion table reported by Wolf22 agreed with the spectrophotometric assay total protein concentrations, refractometer 2 was used as the comparative method for the refractometric difference plots. The biases of refractometric methods for total protein concentrations of serum samples from only 1 dog are reported (Figure 1); similar biases were found in sera from the other dog.

Effects of increased bilirubin concentrations on refractometric total protein concentrations—The measured total bilirubin concentration in the NaOH-spiked serum sample was 0.1 mg/dL. The measured total bilirubin concentrations in bilirubin-spiked samples 1 through 12 were 0.9, 1.7, 2.9, 4.6, 5.6, 6.8, 10.7, 15.1, 21.8, 27.7, 32.7, and 41.5 mg/dL, respectively (Figure 2). Direct bilirubin concentrations in bilirubin-spiked samples 1 through 12 were 0.3, 0.5, 0.4, 0.5, 0.5, 0.6, 0.5, 0.6, 0.7, and 0.8 mg/dL, respectively.

Because the same biases and analytic precision values were consistently found in samples from both dogs, the bilirubin effects on the refractometric total protein concentrations for serum samples from only 1 dog are reported. For that dog, when samples were spiked with only NaOH (1:9 NaOH and serum), thus diluting the unspiked protein concentration, the spiked sample's total protein concentrations were 5.7 g/dL for refractometer 1, 5.4 g/dL for refractometer 2, and 5.5 g/dL for refractometer 3. On the basis of these findings and the refractometric scales being marked at 0.1 g/dL (refractometer 1 and refractometer 3) or 0.1 g/100 g (refractometer 2) intervals, any change to total protein concentration in the bilirubin-spiked samples > 0.2 g/dL was accepted as representing a true change.

![Figure 1](image1.png)

Figure 1—Difference plots of the refractometric total protein concentrations of unspiked and spiked serum samples (n = 14) of 1 of 2 healthy Greyhounds in a study to determine whether increased serum bilirubin concentrations interfere with the measurement of serum total protein concentration by refractometry and to assess potential biases among measurements obtained with 3 temperature-compensated refractometers: a refractometer (refractometer 1) with total protein concentration (g/dL) and refraction scales, a refractometer (refractometer 2) with a TS% scale, and a refractometer (refractometer 3) with a total protein concentration scale (g/dL). Refractometric total protein concentrations determined with refractometer 2 were considered the comparative values (x-axis). For the x-axis, the highest protein concentration (5.8 g/dL) in each graph represents the concentration in the unspiked sample; the lower concentrations (5.1 to 5.4 g/dL) represent samples after they were spiked and thus had diluted protein concentrations. The numbers within circles represent number of values represented by a plotted point. Concentrations for both refractometer 3 (A) and refractometer 1 (B) had positive biases (approx 0.2 g/dL; dashed lines), compared with the refractometer 2 concentrations.

![Figure 2](image2.png)

Figure 2—Line graph of bilirubin concentrations (diamonds) and refractometer 1 (triangles), refractometer 2 (squares), and refractometer 3 (crosses) total protein concentrations in sera of the same dog as in Figure 1 spiked with NaOH only or with increasing amounts of bilirubin up to a total bilirubin concentration of 41.5 mg/dL. The differences in the refractometric total protein concentrations among the refractometers reflect their analytic biases; the variations in concentrations from samples 1 to 12 reflect the methods' analytic precisions.
Table 2—Contributions of major solutes (in various units) to serum TS% (g/100 g × 100%) when there are physiologic concentrations of the solutes.*

<table>
<thead>
<tr>
<th>Solute</th>
<th>Common units</th>
<th>g/dL of serum</th>
<th>g/dL of serum H₂O †</th>
<th>g/100 g ‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total protein</td>
<td>7.0 g/dL</td>
<td>7.0</td>
<td>7.5</td>
<td>7.3</td>
</tr>
<tr>
<td>Glucose</td>
<td>100 mg/dL</td>
<td>0.100</td>
<td>0.107</td>
<td>0.1</td>
</tr>
<tr>
<td>Urea</td>
<td>60 mg/dL</td>
<td>0.060</td>
<td>0.064</td>
<td>0.1</td>
</tr>
<tr>
<td>Na⁺</td>
<td>150 mmol/L</td>
<td>0.345</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>110 mmol/L</td>
<td>0.391</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Na⁺ + Cl⁻</td>
<td>—</td>
<td>0.736</td>
<td>0.794</td>
<td>0.8</td>
</tr>
<tr>
<td>Bilirubin</td>
<td>1 mg/dL</td>
<td>0.003</td>
<td>0.001 &lt; 0.1</td>
<td></td>
</tr>
<tr>
<td>Sum of solutes</td>
<td>—</td>
<td>—</td>
<td>8.4</td>
<td>—</td>
</tr>
<tr>
<td>TS%</td>
<td>—</td>
<td>—</td>
<td>8.2</td>
<td>—</td>
</tr>
</tbody>
</table>

*Original solute concentrations and the calculated TS% are reported with appropriate significant figures. Excess figures are present in some calculated values so that minor changes are seen.  †Value in column derived by dividing value in previous column by 0.938. Converting solute weight per 102.2 g of solution (solute weight + H₂O weight) to solute weight per 100 g of solution. ‡Equals a urea nitrogen concentration of 28 mg/dL or 10 mmol/L. §Conversion of the common clinical concentrations of any solute. The clinical refractometer measures a solute's refractive index, which is used to determine the solution's refractometer, and thus it is useful to rephrase the equation in that context: 

R = r × TS%

Different solutes have different refractivity values, but the major solutes of serum have similar values (human serum protein, R = 19; urea, R = 15; glucose, R = 14; and sodium chloride, R = 17). A refractivity value for bilirubin was not found in the literature. Accordingly, change in serum refractive index is mostly attributable to change in the TS%. In serum or plasma, the largest portion (by weight) of the total soluble solids is the protein content. Thus, proteins are the major contributors to the refraction of serum or plasma.

To understand the relative contribution of solutes to TS%, it is necessary to express concentrations of common serum solutes in terms of their weights and not other common units of concentration. The sum of the solutes' ratio (wt/wt) expression × 100% represents the total protein concentration. When total protein concentration is 7.0 g/dL, the serum water concentration is 93.8 g/dL.22

Discussion

This study found that total bilirubin concentrations up to 41.5 mg/dL did not affect refractometric estimations of serum total protein concentrations, in that there were no detected differences in samples 1 to 12. The minor variations in concentrations obtained with each refractometer reflected the analytic precision of the methods. On the basis of the measured concentrations, nearly all the bilirubin in the spiked samples was considered unconjugated bilirubin. The apparent presence of direct bilirubin (ie, conjugated or delta bilirubin) may have reflected unconjugated bilirubin reacting in the direct diazo reaction.*

The refraction of light by serum is affected by the concentrations of some solutes (eg, proteins, glucose, urea, electrolytes, and lipoproteins). However, even with an extreme increase in serum bilirubin concentration, the calculated increase in TS% is too small (ie, < 0.1%) to affect the serum's refractive index and thus would not falsely increase the refractive total protein concentration. This calculated prediction was confirmed by the measurements of this study. The bilirubin powder used to spike sera contained unconjugated bilirubin, as determined by evaluation of the total and direct bilirubin concentrations, yet none of the various forms of bilirubin should affect the TS%.

When it is stated that hyperbilirubinemia causes a falsely increased refractometric total protein concentration, that statement is in error, and the reason for the

The refractive index of a liquid is proportional to TS%. This key concept is based on a property of solutes called refractivity (R) that is calculated with the following formula:
error is usually unclear but might be based on concepts of photometry. Bilirubin is a pigmented compound that will absorb light wavelengths near 455 nm. However, for clinical refractometry, a broad range of visible light wavelengths (approx 380 to 740 nm) enter the refractometer and only a narrow band of wavelengths are absorbed by the bilirubin. Thus, most of the visible light is not affected by the pigment and it does not affect the measured refraction.

Total protein concentrations derived for refractometer 2 (with the conversion table reported by Wolf[2]) agreed poorly with the concentrations derived for refractometer 1 and refractometer 3 and with other methods of converting refractive index values. However, the increasing bilirubin concentrations did not affect the biases. This study's data provide evidence that the refractometric total protein concentration is dependent on the method of converting a refractive index value to a total protein concentration.

References

a. Sigma-Aldrich, St Louis, Mo.
b. TS Meter Refractometer, model 10400A, Leica Microsystems Inc, Buffalo, NY.
c. TS Meter Refractometer, model 10400B, Leica Microsystems Inc, Buffalo, NY.
d. Veterinary Total Solids Refractometer, model 10436, Leica Microsystems Inc, Buffalo, NY.
e. Cobas c501, Roche Diagnostics, Indianapolis, Ind.