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Negative interference of icteric serum on a bichromatic biuret total protein assay

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Short title: Icteric serum's interference on biuret assay

Abstract

Background: Bilirubin is stated to be a negative interferent in some biuret assays and thus could contribute to pseudohypoproteinemia in icteric samples.

Objective: The purpose of the study was to evaluate the magnitude of and reason for a falsely low total protein concentration in icteric serum when the protein concentration is measured with a bichromatic spectrophotometric biuret assay.

Methods: Commercially available bilirubin was dissolved in 0.1 M NaOH and then mixed with sera from 2 dogs to achieve various bilirubin concentrations of up to 40 mg/dL (first set of samples) and 35 mg/dL (second set of samples, for confirmation of first set of results and to explore the interference). Biuret total protein and bilirubin concentrations were determined with a chemistry analyzer (Cobas® 6000 ~~Cobas~~with c501 module). Line graphs were drawn to illustrate the effects of increasing bilirubin concentrations on the total protein concentrations. Specific spectrophotometric absorbance readings were examined to identify the reason for the negative interference.

Results: High bilirubin concentrations created a negative interference in the Cobas ~~c501~~ biuret assay. The detectable interference occurred with a spiked bilirubin concentration of 10.7 mg/dL in one set of samples and 20.8 mg/dL in a second set. The interference was due to a greater secondary absorbance reading at the second measuring point in the samples spiked with bilirubin, which possibly had converted to biliverdin.

Conclusion: Marked hyperbilirubinemia is associated with a falsely low serum total protein concentrations when measured with a bichromatic spectrophotometric biuret assay. This can result in pseudohypoproteinemia and pseudohypoglobulinemia in icteric serum.

Keywords: bilirubin, clinical chemistry, error, hypoproteinemia

Introduction

There are 2 common analytical methods of determining plasma or serum total protein concentrations: biuret spectrophotometric assays and refractometry. A recent case report described a pseudohypoproteinemia in markedly icteric serum when the serum was assayed with a biuret total protein assay.[1] However, the bilirubin concentration at which the interference becomes detectable was not established. In the case report, the refractometric total protein concentration did not appear to be adversely affected by the hyperbilirubinemia. A recent report established that serum bilirubin concentrations as high as 41 mg/dL do not affect the estimation of total protein concentrations by refractometry.[2]

The biuret method is the most common spectrophotometric method of measuring serum total protein concentration. The biuret reaction depends on the number of peptide bonds in the sample; thus, the protein concentration in the sample. In the reaction, proteins form complexes with copper in alkaline solutions and create a blue-purple product; the amount of product is measured with spectrophotometry. The method is named *biuret method* because a similar copper reaction occurs with the organic compound *biuret,* a condensation product of 2 urea molecules. Some package inserts state that bilirubin can interfere with the biuret assay, but the magnitude of interference or reason for the interference is not typically stated.

Even though the method's chemical principle is constant, there are assay variations used by manufacturers of the biuret reagents. The package inserts for 2 biuret assays (Roche Cobas' c501 and Roche Hitachi module P, Roche Diagnostics, Indianapolis, IN, USA) state that an icteric index up to 20 (approximately 20 mg/dL or 342 µmol/L) and 21 (approximately 21 mg/dL

or 359 μmol/L), respectively, have no significant effects (ie, protein recovery is expected to be within 10 % of the initial value). In another biuret method (Roche Cobas Integra 400/800), the package insert states no significant bilirubin interference (ie, error < 10 %). For another company's biuret assay (Beckman-Coulter, Brea, CA, USA), the package insert states that a bilirubin concentration of up to 40 mg/dL (684 μmol/L) has no significant effect (ie, error < 10 %) on the results. For another biuret assay (Vitros, Ortho Clinical Diagnostics, Rochester, NY, USA), the package insert states a maximum observed bias of 3 % with bilirubin concentrations of 15 mg/dL (256 μmol/L), but states possible test-to-test variation. A recent study indicated an icteric index of 14 causes a decreased total protein concentration when measured with the Cobas 6000 assay, but the magnitude of interference was not stated.[3] Occasionally, it is also stated that icterus causes a positive interference with the biuret assay and results in falsely increased protein measurements.[4]

This study evaluated the effect of adding increasing amounts of bilirubin on a biuret serum total protein assay.

Materials and methods

Blood collection

For each set of samples, venous blood was collected from a healthy, fasted Greyhound dog with syringes and then transferred to evacuated tubes. After approximately 30 min, tubes were centrifuged and nonlipemic and nonhemolyzed sera were harvested.

Bilirubin stock solution and preparing icteric sera

A bilirubin stock solution of 400 mg/dL was prepared by dissolving 40 mg of bilirubin powder (Sigma-Aldrich, St. Louis, MO) in 10 mL of 0.1 M sodium hydroxide (NaOH).[5,6] Serial dilutions of the bilirubin stock solution with 0.1 M NaOH were mixed with constant proportions of sera (1 part bilirubin solution + 9 parts serum) to create bilirubin concentrations between 1 and 40 mg/dL (17 and 684 μmol/L) in the first set of spiked sera, and between 4 and 35 mg/dL (68 and 598 μmol/L) in the second set of spiked sera. The first set of spiked sera was assayed to document the interference; the second set was assayed to confirm findings of the first set, and to explore the reason for the interference. Control sera were prepared by mixing with 0.1 M NaOH solution in comparable proportions. Based on a previous study using the same preparation methods, nearly all bilirubin in the spiked sera was unconjugated.[2]

Assays for serum total protein, total bilirubin, and direct bilirubin concentrations

Clinical chemistry assays were completed on the day of blood collection using an automated chemistry analyzer (Cobas® 6000 with ~~Cobas~~ c501 module, Roche Diagnostics, Indianapolis, IN, USA) and the manufacturer's reagents. The analyzer used a reaction monitoring system in which absorbance readings were recorded while the reaction cuvette, located on a rotating reaction disc, passed across the optical path of the instrument's photometer. Each analytical assay had specific timed readings for the calculation of an analyte's concentration.

The total bilirubin assay was a modified Malloy-Evelyn method.[7] Samples with bilirubin concentrations > 35 mg/dL were automatically diluted. Indices for lipemia, hemolysis and icterus were calculated by the chemistry analyzer by using absorbance differences at 3 bichromatic wavelength pairs (700/660 for lipemia, 600/570 for hemolysis, and 505/480 for icterus), and the

icterus index was reported as a number that approximates the total bilirubin concentration (in mg/dL).[8]

The analyzer's biuret total protein assay was a 2-point-end, bichromatic assay (Figure 1),[9] it will be referred to as the bichromatic biuret assay from this point forward; the formulae used to calculate the total protein concentration in a sample are provided (Figure 2).[9] During the previous 5 months and including the weeks of this study, the coefficient of variation (CV) for the bichromatic biuret assay were 1.5 % for commercial level-1 human control serum (mean concentration of 6.0 g/dL, standard deviation 0.1 g/dL) and 1.5 % for commercial level-2 human control serum (mean concentration of 4.4 g/dL, standard deviation of 0.1 g/dL). Based on the assay's analytical precision, any change to biuret total protein concentration in the spiked samples that was > 0.2 g/dL was considered a true change/interference and not a random analytic error.

A refractometer (Leica TS Meter Refractometer Model 10400B) with a total solids percent scale (g/100g) was used to determine refractometric total protein concentrations. The total solids percentage of a sample was converted to a total protein concentration (g/dL) using Wolf's conversion table.[~~9~~10] This method of obtaining a refractometric total protein concentration was selected because it provided the best agreement with the bichromatic biuret assay among 3 available refractometers.[2] The refractometer was calibrated with deionized water to 1.000 on the urine specific gravity scale prior to sample analysis. To monitor analytical precision the day of analyzing the first set of sera, the prespiked serum was analyzed 8 times by refractometry and the bichromatic biuret assay.

The first set of spiked sera was analyzed by refractometry and the bichromatic biuret assay in duplicates, and the mean concentration of each sample was recorded for comparisons.

The second set of prespiked and spiked sera was assayed 5 times, and the mean concentration of each sample was recorded for comparisons. In both sets of samples, the biuret total protein concentrations were evaluated to detect negative interference. Actual absorbance data of the second set of spiked sera were evaluated to determine the reason for the negative interference.

Results

The mean total protein concentration in the first prespiked serum by both methods (refractometry and biuret reaction) was 5.8 g/dL (CV 0.9 % and 1.4 %, respectively).

In the first set of spiked sera, lower bichromatic biuret total protein concentrations (> 0.2 g/dL decrease compared to protein concentration in the NaOH sample) were first seen at a bilirubin concentration of 10.7 mg/dL, corresponding to an icteric index of 16 (Figure 3, sample S7). Whereas, refractometric total protein concentrations ranged from 5.0 - 5.2 g/dL in the same spiked sera (Figure 3).

In the second set of spiked sera, lower bichromatic biuret total protein concentrations occurred at a bilirubin concentration of 20.8 mg/dL (356 µmol/L) (corresponding to an icteric index of 29) and at greater bilirubin concentrations (Figure 4).

The analysis of the actual absorbance data (Table 1) provided additional information for the observed bilirubin interference:

1. At the first measuring point (mp_1), the measured primary absorbance values at 546 nm increased proportional to increasing bilirubin concentrations because bilirubin absorbs the 546 nm light. Minor fluctuations in the secondary absorbance values at 700 nm reflect analytical variation, as the 700 nm light is not absorbed by bilirubin.

2. At the second measuring point (mp_2) and thus at the end of the biuret reaction producing a blue-purple product, the measured primary absorbance values at 546 nm were variable but greatest in the spiked sera with the highest bilirubin concentrations, reflecting that both bilirubin and the product of the biuret reaction were contributing to the primary absorbance values. The secondary absorbance values at 700 nm increase mildly for samples with bilirubin concentrations up to 9.9 mg/dL (169 µmol/L), then more abruptly at 20.8 mg/dL, 31.7 mg/dL, and 36.3 mg/dL (356 µmol/L, 542 µmol/L, and 621 µmol/L).
3. Amp_1 values are the mathematical differences between the measured absorbance values at 546 nm and 700 nm at the first measuring point (mp_1). The difference increased as the bilirubin concentrations increased reflecting the absorbance of the 546 nm light by bilirubin. The Amp_1 value is used in the calculation of the protein concentration (Figure 2). .
4. Amp_2 values are the mathematical differences between the measured absorbance values at 546 nm and 700 nm at the second measuring point (mp_2). Amp_2 values fluctuated with bilirubin concentration of up to 9.9 mg/dL (169 µmol/L), then declined at greater bilirubin concentrations. The decreasing *Amp2* values correspond with the abrupt increases in the secondary absorbance values of at the end of the bichromatic biuret reaction reflecting the presence of an interfering substance. The Amp_2 value is used in the calculation of the protein concentration (Figure 2). Examples of the calculations are shown in Figure 5.
5. A_x values are calculated using the absorbance values from the first and second measuring points (Amp_1 and Amp_2, respectively) (Figure 2). There are minor changes in the A_x values with bilirubin concentration of up to 9.9 mg/dL (169 µmol/L), then decreased abruptly at greater bilirubin concentrations reflecting the appearance of the interfering substance.

6. The lowering of the total protein concentration to 5.0 g/dL from the prespike concentration (5.5 g/dL) reflects the dilution of the protein concentration when the NaOH solution was added. When spiked with the bilirubin solutions, the measured protein concentration varied minimally up to the bilirubin concentration of 9.9 mg/dL (169 µmol/L), and then decreased abruptly at greater bilirubin concentrations reflecting the appearance of the interfering substance.

Discussion

Increasing serum bilirubin concentrations in both sets of spiked sera resulted in a negative interference of the measurement of the total protein concentration by the bichromatic biuret method while the refractometric total protein concentrations remained the same in the first set of spiked samples.

The maximum spectral absorption of bilirubin in serum is near 455 nm,[~~10~~11] therefore, the interfering agent found in the secondary-wavelength (700 nm) absorbance was likely not bilirubin. However, bilirubin can be degraded to biliverdin in an alkaline environment.[~~11~~12] Because the biuret reaction occurred in an alkaline solution, we hypothesize that some of the bilirubin converted to biliverdin, which has maximum spectral absorbance near 700 nm,[~~12,13~~13,14] the secondary wavelength of the evaluated biuret assay. Assays to measure biliverdin concentrations were not attempted and thus this hypothesis was not tested or confirmed. Nevertheless it can be assumed that the degree of interference due to biliverdin or another compound derived from bilirubin will be roughly proportional to the initial amount of bilirubin present in the reaction cuvette. Until the identity and property of such compounds are clarified, it

will be difficult to predict to what degree hyperbilirubinemia will interfere with bichromatic biuret assay.

The presence of a decreased total protein concentration in the presence of marked hyperbilirubinemia could lead to erroneous conclusions if the pseudohypoproteinemia is not recognized. This study documents that marked hyperbilirubinemia can be associated with a false decrease in the serum total protein concentration measured by a bichromatic biuret assay. The interferent is not bilirubin but is related to the presence of bilirubin in the serum. The negative interference was found in one bichromatic, 2-point-end assay; it is not known if a similar interference will be found with other biuret assays.

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Figure Legends

Figure 1: The bichromatic biuret assay (Cobas® 6000 with Cobas c501 module, Roche Diagnostics, Indianapolis, IN, USA) is a 2-point-end spectrophotometric assay. After addition of water (blank) to the reaction cuvette, 3 absorbance readings are taken (C_1, C_2, & C_3). After the addition of the sample (S) and first reagent (R_1) that contains NaOH and a buffer, the first measuring point (mp_1) at the 10th absorbance reading (Amp_1) is obtained. After addition of the second reagent (R_2) that contains NaOH, a buffer, potassium iodide, and copper sulfate and a specified time interval, the second measuring point (mp_2) at the 34th absorbance reading (Amp_2) is obtained. The second measuring point designates the end of the assay. If protein is present in the sample, then Amp_2 is greater than Amp_1. It is a bichromatic assay because 2 absorbance readings are recorded at each measuring point: 1) primary-wavelength absorbance at 546 nm represents the total absorbance of the cuvette; 2) secondary-wavelength absorbance at 700 nm represents any absorbance due to an interfering substance such as lipids. After compensating for dilution in the reaction cuvette at mp_2, the absorbance at mp_1 (Amp_1) is subtracted from absorbance at mp_2 (Amp_2) to determine the change in absorbance due to the product of the biuret reaction. (Figure modeled after figure within Roche Diagnostics literature for its Cobas c501 instrument.[9])

Figure 2: Formulas used to calculate total protein concentration in the bichromatic biuret assay (Cobas® 6000 with Cobas c501 module, Roche Diagnostics, Indianapolis, IN, USA). (Figure

modeled after similar equations within Roche Diagnostics literature for its Cobas c501 instrument.[9])

Figure 3: Means of duplicate measurements of refractometric (··×··) and bichromatic biuret (–□–) total protein concentrations in sera spiked with only NaOH solution or with increasing amounts of bilirubin. The measured bilirubin concentrations (- - ◊ - -) were determined by a modified Malloy-Evelyn method (Cobas® 6000 withCobas c501 module, Roche Diagnostics, Indianapolis, IN, USA). The biuret total protein concentrations were nearly constant up to a total bilirubin concentration of 6.8 mg/dL (116 µmol/L) (sample S6), then decreased in a near linear progression to a total protein concentration of 4.1 g/dL when the bilirubin concentration was 41.5 mg/dL (710 µmol/L). The minor changes in refractometric total protein concentrations were considered to be within the analytical variation and thus high bilirubin concentrations did not interfere with the refractometric estimations of the total protein concentrations.

Figure 4: Means of quintuplicate absorbance values (A_x) (··×··) that were used to calculate biuret total protein concentrations (numerical value of the protein concentration {g/dL} below each plotted point) and mean of duplicate measurements of bilirubin concentrations (- - ◊ - -) (numerical value of icteric index below each plotted point) in sera spiked with only NaOH solution or with increasing amounts of bilirubin. The measured bilirubin concentrations were determined by a modified Malloy-Evelyn method (Cobas® 6000 with Cobas c501 module, Roche Diagnostics, Indianapolis, IN, USA). The biuret total protein concentrations were nearly constant up to a total bilirubin concentration of 9.9 mg/dL (169 µmol/L) (sample S7), then decreased in a near linear progression to a total protein concentration of 4.0 g/dL when the

bilirubin concentration was 36.3 mg/dL (621 µmol/L). The second absorbance values (Amp_2) (‥Δ‥) and first absorbance values (Amp_1) (‥□‥) are plotted for each spiked sample; those values are used to calculate the A_x (Figure 2). When the samples' bilirubin concentrations were 20.8 mg/dL (356 µmol/L, icteric index = 29) (sample S8) and greater, there were obvious decreases in Amp_2 and minor increases in (Amp_1) which results in a lower calculated A_x. These absorbance changes resulted in falsely low total protein concentrations.

Figure 5: Calculation of total protein concentrations in "NaOH only" sample and "S8" serum samples in the second set of sera (see Table 1 for sources of absorbance values)

Table 1: Total bilirubin concentrations and absorbance values in the bichromatic biuret serum total protein assay of the chemical analyzer (Cobas® 6000 with ~~Cobas~~ c501 module) with bilirubin spiked normal canine sera

Sample	[Total bilirubin] (mg/dL)	Absorbance data							
		mp_1		mp_2		Amp_1	Amp_2	A_x	Biuret [total protein] (g/dL)
		1°A[‖]	**2°A****	**1°A**	**2°A**	**1°A – 2°A**	**1°A – 2°A**		
Prespike	0.1	0.0000	-0.0006	0.2342	0.1241	0.0006	0.1101	0.1097	5.5
Controls (NaOH only)	0.1	0.0005	-0.0002	0.2168	0.1236	0.0007	0.0932	0.0927	5.0
S1	4.0	0.0011	-0.0004	0.2140	0.1282	0.0015	0.0858	0.0847	4.9
S2	4.9	0.0016	-0.0003	0.2161	0.1293	0.0019	0.0868	0.0854	4.9

S3	6.0	0.0026	0.0004	0.2195	0.1296	0.0022	0.0899	0.0883	4.9
S4	7.2	0.0023	-0.0004	0.2183	0.1319	0.0027	0.0864	0.0844	4.7
S5	8.4	0.0030	-0.0002	0.2218	0.1319	0.0032	0.0899	0.0875	4.8
S6	9.4	0.0030	-0.0003	0.2175	0.1334	0.0033	0.0841	0.0817	4.7
S7	9.9	0.0033	-0.0001	0.2208	0.1331	0.0034	0.0877	0.0852	4.8
S8	20.8	0.0071	0.0004	0.2208	0.1439	0.0067	0.0769	0.0719	4.4
S9	31.7	0.0099	0.0007	0.2223	0.1522	0.0092	0.0701	0.0633	4.2
S10	36.3	0.0120	0.0006	0.2262	0.1586	0.0114	0.0676	0.0591	4.0

[*] $mp_{1:}$ first measuring point of primary and secondary absorbance values, reaction cuvette contains water, sample, and the first reagent.

[†] $mp_{2:}$ second measuring point of primary and secondary absorbance values, the reaction cuvette contains water, sample, first reagent, and second reagent; biuret reaction completed

[‡] Amp_1 = difference of primary and secondary absorbance values at mp_1.

[§] Amp_2 = difference of primary and secondary absorbance values at mp_2.

[¶] A_x = the calculated absorbance value (see Figure 2) that is used to calculate a sample's total protein concentration

[‖]1°A is the primary absorbance for the biuret assay at 546 nm which represents total absorbance by the reaction cuvette.

[**]2°A is the secondary absorbance for the biuret assay at 700 nm which represents absorbance due to interfering substances.

Images

Figure 1

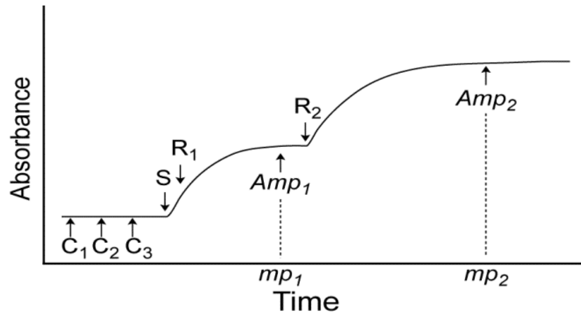

Figure 2

1) $A_x = Amp_2 - d \times Amp_1$

$$\text{where } d = \frac{V_{sample} + V_{R1}}{V_{sample} + V_{R1} + V_{R2}}$$

A_x is absorbance value for calculation of total protein concentration

Amp_2 is absorbance (primary - secondary) @ 34th measurement point

Amp_1 is absorbance (primary - secondary) @ 10th measurement point

d is dilution factor (adjusting Amp_1 because the Amp_2 is read after addition of reagent 2)

V_{sample} is sample volume used in the assay

V_{R1} is the volume of reagent 1

V_{R2} is the volume of reagent 2

2) $C_x = [K(A_x - A_b) + C_b] \times (IF_A + F_B)$

C_x is calculated total protein concentration of sample

K is calibration factor for current calibrator of biuret assay

A_x is absorbance value for calculation of total protein concentration

A_b is absorbance value of blank calibrator

C_b is total protein concentration in blank calibrator

$IF_A + F_B$ are the instrument constants for a slope of 1 and intercept of 0

Figure 3

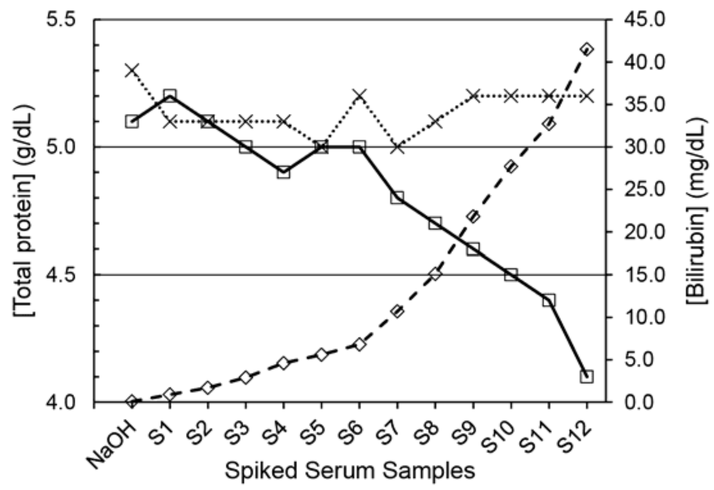

Figure 4

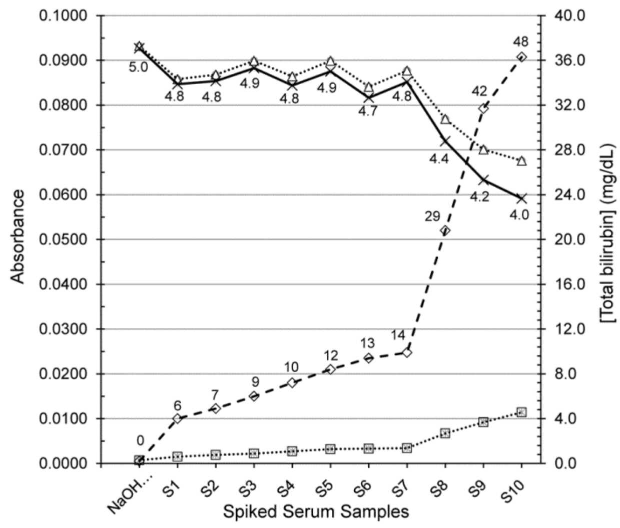

Figure 5

When $V_{sample} = 2\ \mu L; V_{R1} = 90\,\mu L; V_{R2} = 32\,\mu L$

$$d = \frac{V_{sample} + V_{R1}}{V_{sample} + V_{R1} + V_{R2}} = \frac{2\,\mu L + 90\,\mu L}{2\,\mu L + 90\,\mu L + 32\,\mu L}$$

$= 0.7419$

Using absorbance values of NaOH sample in Table 1 and formulae in Figure 2

$A_x = Amp_2 - d \times Amp_1 = 0.0932 - 0.7419 \times 0.0007$

$= 0.0927$

When $K = 29.6$, $A_b = -0.0773$, $C_b = 0.0$ g/dL

and $(IF_A + F_B) = 1 + 0$

C_x of NaOH sample = $[K(A_x - A_b) + C_b] \times (IF_A + F_B)$

$= [29.6(0.0927 + 0.0773) + 0.0] \times 1.0$

= 5.0 g/dL

Using absorbance values of S8 sample in Table 1 and formulae of Figure 2

$A_x = Amp_2 - d \times Amp_1 = 0.0769 - 0.7419 \times 0.0067$

$= 0.07193$

When $K = 29.6$, $A_b = -0.0773$, $C_b = 0.0$ g/dL

and $(IF_A + F_B) = 1 + 0$

C_x of S8 = $[K(A_x - A_b) + C_b] \times (IF_A + F_B)$

$= [29.6(0.07193 + 0.0773) + 0.0] \times 1.0$

$= 4.4\ g/dL$