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ARTICLE

False Positive Acetaminophen Levels Associated with Hyperbilirubinemia

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Serum acetaminophen determination is frequently necessary in patients with hepatic failure. We observed two patients (#1, #2) with elevated serum total bilirubin levels (26.5 mg/dL and 40.1 mg/dL) who had multiple false positive acetaminophen levels using the kinetic method of the GDS Diagnostics enzymatic acetaminophen assay (GDS Diagnostics, Elkhart, IN). We investigated the magnitude, threshold, and linearity of this effect using the GDS Diagnostics assay and an EMIT acetaminophen assay on two other hyperbilirubinemic patients (#3, #4) and a commercial solubilized bilirubin standard. Samples were diluted using fresh frozen plasma, and acetaminophen levels were analyzed twice using the kinetic method of the GDS Diagnostic acetaminophen assay and twice with the EMIT assay. The absence of acetaminophen in all samples was verified by gas chromatography/mass spectroscopy (GC/MS). The kinetic GDS assay resulted in a positive acetaminophen assay (cutoff for a positive result = 10 mg/L) with patient #3, patient #4, and in the bilirubin standard when the total bilirubin levels were 28.2 mg/dL, 22.5 mg/dL, and 18.3 mg/dL, respectively. One sample was interpolated to give a positive acetaminophen reading when diluted to a total bilirubin concentration of 15 mg/L. None of the samples tested with GC/MS or the EMIT assay resulted in any detectable acetaminophen. In conclusion, caution must be taken utilizing the GDS Diagnostic assay for the quantification of acetaminophen with concomitant hyperbilirubinemia. Alternatives such as EMIT or GC/MS should be employed to assess acetaminophen levels in such patients.

Keywords GDS; EMIT; GC/MS; Bilirubin; Analytical

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INTRODUCTORY STATEMENT

Acetaminophen toxicity is one of the leading causes of fulminant hepatic failure (1). Patients presenting with evidence of hepatic dysfunction are usually screened for the presence of serum acetaminophen. A detectable acetaminophen level has important ramifications for treatment and transplant decisions. There are two broad assays for acetaminophen detection in common clinical use. One relies on an enzymatic reaction with acetaminophen forming a chromophore; the GDS Diagnostics (GDS Diagnostics, Elkhart, IN) method is one such method. Another assay utilizes acetaminophen specific antibodies, with the EMIT® tox™ Acetaminophen (Dade Behring, Cupertino, CA) assay being an example of several antibody techniques which also include turbidimetric and fluorescence polarization.

The GDS manufacturer recommends the assay may be used in two different ways; a static (end point) method that assesses light absorption after 10 min, and a kinetic method that determines the rate of change of light absorption repeatedly from 3 to 5 min. The manufacture of the GDS assay states that bilirubin concentrations of 25 mg/dl cause elevations of measured acetaminophen levels in the presence of acetaminophen, but does not distinguish between the method (static or kinetic) with this caution, and false positive rates were not reported (2). Bertholf et al. (3) reported false positive acetaminophen measurements with hyperbilirubinemic serum samples as low as 8 mg/dl when employing a modified GDS method using two point rate determination with read points at only 13 and 19 sec. It was hypothesized that if the GDS method utilized a longer time between read points this interference might be avoided.

We observed falsely positive acetaminophen concentrations utilizing the manufacturer's recommended kinetic GDS method with a total analysis time of 2 min (patients #1 and #2, Table 1). In several samples from the two patients, the serum

TABLE 1
Values of acetaminophen observed in hyperbilirubinemic patients

Laboratory values	Patent #1 Hours post-admission				Patient #2 Hours post-admission		Patient #3	Patient #4	Bilirubin standard
	16	41	210	282	306	346			
Bilirubin, mg/dL	17.7	23.3	21.0	26.5	32.7	40.1	49.6	37.2	32.7
Direct bilirubin, mg/dL	–	–	14.2	–	23.2	26.3	–	–	–
Acetaminophen, mg/L	22	16	22	26	28	26	5	26.9	29.1
By GDS method Acetaminophen, GC/MS	None	None	None	None	None	None	None	None	None

bilirubin concentrations in two hyperbilirubinemic patients were below the manufacture's reported interference threshold. The absence of acetaminophen in all samples was confirmed by gas chromatography/mass spectroscopy (GC/MS). The purpose of this study, then, was to investigate the magnitude, threshold, and linearity of the interference of hyperbilirubinemia on the kinetic GDS assay and to compare results with an EMIT acetaminophen assay.

EXPERIMENTAL PROCEDURES

Serum Acetaminophen Measurements

The two methods used for acetaminophen quantitation were the GDS Diagnostic assay and the EMIT® tox™ acetaminophen assay. Both procedures were carried out on a Hitachi 911 automated analyzer.

The GDS Diagnostics assay utilizes n-arylacylamidase to cleave acetaminophen to p-aminophenol and acetate. The p-aminophenol then reacts with o-cresol in the presence of periodate to form the chromophore indophenol which has a strong absorption at 615 nm. The manufacturer recommends performing this as either a static (end point) or kinetic method. We used the kinetic method, which measured the change in optical density at 615 nm over time beginning 3 min after reaction initiation, and measuring absorbance every 10 sec over a 2 min period. The resulting slope is proportional to the acetaminophen concentration (2). The static method uses one absorbance reading at 10 min, which is proportional to the acetaminophen concentration.

The EMIT® tox™ assay utilizes heterogeneous antibodies to acetaminophen. A mixture of the antibodies and nicotinamide adenine dinucleotide (NAD) is combined with the sample. Then, acetaminophen labeled with glucose-6-phosphate dehydrogenase is added. The greater the amount of acetaminophen in the patient's serum, the less binding of the labeled acetaminophen occurs and the greater the conversion of NAD to NADH. The method is a rate reaction (two measurments between 19 and 23 sec), and the change in absorbance at 340 nm over time is proportional to the acetaminophen concentration (4).

GC/MS was used to verify the absence of acetaminophen in patient samples, fresh frozen plasma (FFP), and bilirubin standard. For GC/MS analysis, acetaminophen was extracted by mixing 100 µl of serum into 4.5 mL of pH 6.0 phosphate buffer and mixing with salted ethyl acetate/methylene chloride (toxi-lab A tube, Ansys, Harbor City) along with 100 µl of mepivacaine as the internal standard (1 mg/L). The organic layer was evaporated down and then reconstituted into ethyl acetate before injection into a Hewlett-Packard GC/MS using a DB5-MS column. The GC/MS analytical detection limit was verified using serial dilutions of FFP with acetaminophen standards and was determined to be below 5 mg/L.

Specimens and Dilutions

The bilirubin standard employed was Bilirubin Lin-Trol® (Sigma Diagnostics, St. Louis) with total bilirubin of 32.7 mg/dL. Serum from two other patients, patient #3 with total bilirubin of 49.6 mg/dL and patient #4 with total bilirubin of 37.2 mg/dL, were studied. The samples were without patient identifiers and were analyzed approximately 1 month following discharge after being stored frozen. The samples were diluted using expired FFP. The dilutions (6) were made

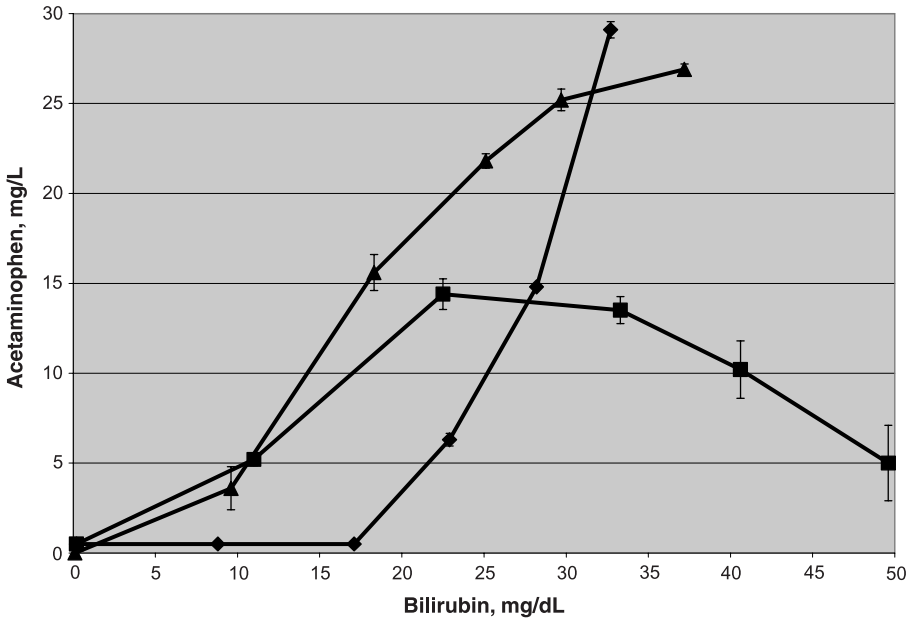

FIG. 1. Comparison of measured acetaminophen levels with total bilirubin levels. (◆) represents hyperbilirubinemic standard, (■) represents patient #3 (bilirubin of 49.6 mg/dL), and (▲) represents patient #4 (bilirubin of 37.2 mg/dL). Data points represent mean values (N=2 for each data point)±S.E.M.

using 0, 130 µl, 250 µl, 350 µl, 430 µl, and 500 µl of the hyperbilirubinemic specimens and brought up to a total volume of 500 µl using FFP. Each dilution was split into three disposable analyzer wells. One GDS and EMIT measurement was made on each of two wells, and the third well was utilized for bilirubin quantification. This resulted in a total of 12 GDS and 12 EMIT measurements (2 at each dilution) on each of the three samples.

Statistical Analysis

Average values of the GDS and EMIT results were determined for each dilution (n=2 for each data point). The average values for the GDS acetaminophen concentration vs. bilirubin concentration were graphed. The results were clearly not linear, and polynomial regression analysis was used to determine the best line fit, determined by the value of R^2.

RESULTS

All patient samples, including FFP and the bilirubin standard were free of acetaminophen as determined by GC/MS. The GDS assay resulted in false positive acetaminophen levels once a threshold bilirubin concentration was reached in all samples; see Fig. 1 and Table 1. In patient #4 at a bilirubin level of 18 mg/dL, the acetaminophen level exceeded 10 mg/L, which is the low cutoff value for the GDS assay (2). One can extrapolate that a positive acetaminophen level could occur with a bilirubin as low as 15 mg/dL if the interference was linear between the data points. This interference level is well below the 25 mg/dL bilirubin concentration previously reported to interfere with the GDS assay (2). Interestingly, patient #3 demonstrated an interference with the GDS assay which peaked at a 1:1 dilution (bilirubin of 22.5 mg/dL) with an acetaminophen level of 14.4 mg/L. The interference subsequently decreased as the sample became more concentrated to a false positive acetaminophen level of only 5 mg/L at a bilirubin of 49.6 mg/dL. The bilirubin standard and patients #3 and #4 did not result in a detectable acetaminophen level using the EMIT assay.

The best fit for each sample's curve was a nonlinear cubic polynomial equation. However, these equations were quite different for each specimen and no comparisons could be made.

DISCUSSION

Although hyperbilirubinemia has been previously reported to interfere with the GDS acetaminophen assay, this study differs in important ways. Bertholf et. al. explored the effect of sample dilution with saline and analyzed a hyperbilirubinemic sample spiked with acetaminophen (3), while the samples in this study were analyzed with GC/MS to verify the absence of acetaminophen, and all dilutions were performed using acetaminophen free human fresh frozen plasma instead of saline to closely approximate physiologic conditions. Bertholf used a modified rate method, but with measurements only at 13 and 19 sec. We used an analysis time that was 20 times longer, which had been hypothesized to limit interference by bilirubin in the assay (3). However, even with longer measurement times using the kinetic GDS Diagnostics assay, we found hyperbilirubinemia may produce false positive acetaminophen concentrations at serum bilirubin concentrations

well below the 25 mg/dL value found in the manufactuer's cautionary statement.

The mechanism of this interference is unknown. A reaction of the bilirubin with the o-creosol, generating a compound with absorbance at 615 nm, would seem to be the most likely cause. In the absence of such a reaction between bilirubin and o-cresol, the baseline absorbance of bilirubin should not affect the kinetic GDS method, since the analysis measures the change in absorbance over time, not the absolute absorbance. Furthermore, if bilirubin was the only reason for the interference, the absorbance should be linearly related to its concentration. However, we demonstrated a nonlinear interference relationship with the bilirubin concentration. One sample (patient #3) experienced a maximal interference with a bilirubin of 22.5 mg/dL, with decreasing response as the bilirubin concentration increased. This trend also appeared with the 12 nondiluted hyperbilirubinemic samples reported by Bertholf et al. (3) If the arylacylamidase participates in this side reaction, the enzyme likely has poor affinity for bilirubin because little interference was observed in the presence of acetaminophen (3). It is possible that this interference from hyperbilirubinemia could occur with other colorimetric acetaminophen assays that rely on absorption around 615 nm.

The nonlinearity of this side reaction does not appear to be due to exhaustion of the reagents, as little effect was previously reported on acetaminophen analysis when 150 mg/L acetaminophen was added to a hyperbilirubinemic sample (3). One reason for the nonlinearity could be due to the limitations of Beer's law (absorbance is proportional to concentration). All of the samples had relatively high absorbances. With a concentrated solution increased turbidity, intermolecular interactions, and increased dark current contribute to nonlinearity.

Despite absence of acetaminophen, hyperbilirubinemia was associated with measurable acetaminophen concentrations using the kinetic GDS Diagnostics assay method, even with bilirubin concentrations below 25 mg/dL and 2 min analysis times. The mechanism and threshold of this interference remain unknown. The EMIT assay did not demonstrate this interference from hyperbilirubinemia. Clinicians must realize elevated acetaminophen levels in hyperbilirubinemic patients using the GDS assay should not always be interpreted as evidence of acetaminophen use.

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