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Characterization of bilirubin interference in three commonly used digoxin assays

Paul D. Simonson[a], Katie H. Kim[a], Gabrielle Winston-McPherson[a], Rugvedita S. Parakh[a], Diane Yamaguchi[a], Anna E. Merrill[b], Jane A. Dickerson[a,c], Dina N. Greene[a,*]

[a] Department of Laboratory Medicine, University of Washington, Seattle, WA, United States
[b] Department of Pathology, University of Iowa, Iowa City, IA, United States
[c] Department of Pathology, Seattle Children's Hospital, Seattle, WA, United States

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ABSTRACT

Background: Due to the narrow therapeutic range of digoxin, determining serum/plasma digoxin concentrations is critical for assessing patients with congestive heart failure, atrial fibrillation, and certain types of arrhythmias. However, digoxin quantification by competitive immunoassays is susceptible to interferences that may alter the accuracy of its measurement in patient plasma. This study aimed to characterize the extent of bilirubin interference in three commonly used digoxin immunoassays.

Methods: Digoxin concentrations were compared using the Beckman Coulter® Unicel DxI 800, the Vitros® 4600, and the Roche Cobas® 8000 in neat or digoxin-spiked icteric and non-icetric plasma samples. A mixing study was performed to demonstrate how digoxin quantification is affected by bilirubin. An equation was derived that predicts the response of the DxI 800, given known bilirubin and digoxin concentrations.

Results: The DxI reported detectable concentrations of digoxin in high bilirubin samples with no added digoxin, while the Vitros® 4600 and Cobas® 8000 gave virtually undetectable results. Spiking digoxin into samples with elevated bilirubin concentrations resulted in a higher percent recovery for the DxI 800 when compared to the other two platforms. The mixing study also revealed an increase in the percent recovery in the DxI 800, while the Vitros® 4600 and Cobas® 8000 were comparable to the expected concentration of digoxin.

Conclusions: The DxI 800 is most prone to interference by bilirubin, while the Vitros® 4600 and Cobas® 8000 are relatively unaffected. Icteric samples should be interpreted with caution if digoxin quantification is needed, especially on the DxI 800 assay.

1. Introduction

Digoxin is an important cardiac glycoside used to manage congestive heart failure and certain types of arrhythmias, as well as control the ventricular rate in patients with atrial fibrillation. Due to its very narrow therapeutic range, drug monitoring of digoxin is critical to ensure that patients being treated with digoxin avoid toxicity [1,2]. The implications of incorrectly reporting digoxin concentrations are substantial; digoxin toxicity can lead to cardiac disturbances, gastrointestinal problems, and central nervous system disorders. Higher digoxin concentrations correlate with more disturbances. Awareness of the pitfalls of digoxin testing, including how bilirubin affects reported digoxin concentrations, is important for physicians and laboratorians to help ensure the safety of often critically ill patients.

Digoxin can be quantified by competitive immunoassays using various commercial, multifunctional analyzers. While similar in overall approach, the assays use different labels, analyte separation methods, and detection methods. Labeled digoxin (digoxin coupled to enzyme or catalyst) is added to the sample and competes with unlabeled digoxin for binding sites on the antibody. Next, the sample is washed to remove unbound digoxin. Finally, substrate is added to react with the labeled digoxin, and the patient's digoxin concentration is inversely proportional to the amount of converted substrate that is detected. The Unicel DxI 800 (Beckman Coulter, Inc., Fullerton, CA) is one such analyzer that utilizes this detection method [3].

Unfortunately, bilirubin is known to interfere with digoxin quantification on the DxI 800 [3], though the extent of the interference is not generally known. Some common causes of elevated bilirubin concentrations in patient plasma include liver disease, cirrhosis, uremia, and bile duct obstruction. Ignoring the effect of elevated bilirubin may cause a misinterpretation of digoxin results by physicians, putting patients at serious risk for harm.

* Corresponding author.
E-mail address: dngreene@uw.edu (D.N. Greene).

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The objective of this study was to compare how an elevated bilirubin concentration affects digoxin quantification on three different analyzers in order to better inform interpretation of digoxin on the DxI 800 instrument. This was accomplished by comparing digoxin concentrations in icteric, non-icteric, and mixed samples. Overall, our data demonstrate that the DxI is most prone to interference by bilirubin, reporting higher than expected digoxin concentrations, while the Vitros® 4600 (Ortho-Clinical Diagnostics, Inc., Rochester, NY) and the Cobas® 8000 by Roche (Roche Diagnostics, Indianapolis, IN) are relatively unaffected.

2. Materials and methods

2.1. Assays

Digoxin concentrations were measured using a Beckman Coulter DxI 800. Bilirubin concentrations were measured using a Beckman Coulter AU680 [4]. Samples were also evaluated for digoxin concentration using the Vitros® 4600 (Seattle Children's Hospital) and Roche Cobas® 8000 (University of Iowa). Each competitive immunoassay method varied slightly in the analytical measurement ranges (AMR), therapeutic range, and precision (Table 1) [3,5,6]. Though each analyzer uses the same overall approach (competitive immunoassay), the differences in their antibodies and detection methods may produce slightly different results.

The Beckman Coulter DxI 800 assay measures digoxin concentrations by competition between modified (alkaline phosphatase coupled) digoxin and unmodified digoxin (from the patient's blood) for a limited, known number of (rabbit polyclonal) antibody binding sites. After binding, the sample is washed, with retention of the digoxin by paramagnetic particles coupled to anti-digoxin antibodies. The resulting bound digoxin-coupled phosphatase reacts with a chemiluminescent substrate (Lumi-Phos 530), and the resulting chemiluminescence is measured. The package insert for the instrument states that interference with digoxin measurements occurs at total bilirubin concentrations of 10.0 mg/dL; however, further details of the nature of the interference are lacking.

The Vitros® 4600 assay is also a competitive immunoassay; however, this assay uses monoclonal antibodies and dry slide technology [7,8]. The displaced digoxin is coupled to a different enzyme (horseradish peroxidase), and the enzyme substrate is a leuco dye, with rate of leuco dye formation measured by reflectance spectrophotometry at 670 nm [9].

The Roche Cobas® 8000 assay uses monoclonal antibodies coupled to streptavidin-coated nanoparticles. The competing digoxin is coupled to ruthenium, and concentration is measured via an electrochemiluminescent assay.

2.2. Biological samples

Non-icteric samples were prepared using residual plasma from patients receiving digoxin (n = 10). These samples were aliquoted, frozen, and later assayed using all three assays.

For the spiking study, pools of patient plasma (n = 2) with known elevated bilirubin concentrations were prepared. Each pool was made using residual plasma from a single patient who was not receiving digoxin. These plasma pools were spiked with known amounts of pharmaceutical digoxin (Westward Digoxin, USP 500 μg/2 mL) at three different concentrations.

For the mixing study, one part plasma from known high digoxin samples was mixed with one part plasma from known high bilirubin samples. Samples were selected so that, following dilution, the digoxin concentration was kept within the assay linear range (above the limit of quantitation). The resulting mixtures had bilirubin concentrations > 10 mg/dL. Digoxin concentration was measured, and the percent recovery of digoxin was calculated from each of the three assays.

Since analysis was performed as quality improvement, institutional review board approval for use of patient samples was not required.

Table 1
Digoxin assay comparisons.

Analyzer	AMR (ng/mL)	Digoxin therapeutic range (ng/mL)	Quality control level	Mean	% CV (per institution)
Beckman Coulter® Unicel DxI 800	0.2–6.0	0.5–1.9	1	0.97	5.30%
			3	3.4	4.40%
Vitros® 4600	0.4–4.0	0.5–2.0	1	1.1	9.10%
			2	2.7	7.40%
Cobas® 8000	0.4–5.0	0.5–2.0	1	0.80	3.80%
			3	3.5	2.50%

2.3. DxI 800 results modeling

The reported digoxin concentrations were modeled as a function of the known digoxin and bilirubin concentrations as

$$z = a\,[bilirubin] + b\,[digoxin] + c \tag{1}$$

where z is the reported digoxin concentration (ng/mL), bilirubin concentration (mg/dL), actual digoxin concentration (ng/mL), and a, b, and c are fitting parameters. Fitting was performed using least-squares using the *Python 2.7 scipy* library. Solving for actual digoxin concentration as a function of reported digoxin concentration and known bilirubin concentration yields

$$[digoxin] = (z - c - a\,[bilirubin])/b \tag{2}$$

with estimated uncertainty [10]

$$s_{[digoxin]} = \sqrt{\frac{(z-c-[bilirubin])^2}{b^2}s_a^2 + \frac{(z-c-a[bilirubin])^2}{b^4}s_b^2 + \frac{(z-1-a[bilirubin])^2}{b^2}s_c^2 + \frac{(1-c-a[bilirubin])^2}{b^2}s_z^2 + \frac{(z-c-a)^2}{b^2}s_{[bilirubin]}^2} \tag{3}$$

where the s values correspond to the estimated standard errors associated with each of their corresponding fit, reported, or known values.

3. Results

The inter-instrument comparisons of digoxin concentrations in non-icteric specimens demonstrated that in patients with normal concentrations of bilirubin who were receiving digoxin, both the Vitros® and the Cobas® compared well with the DxI for digoxin concentrations < 0.4 ng/mL, considering that the reportable range for the two assays had a lower cutoff of 0.4 ng/mL digoxin (Table 2). For values above 0.4 ng/mL, the DxI 800 and Vitros® 4600 produced similar results, but the Cobas® 8000 results were 16% lower than the DxI 800 baseline value.

Spiking elevated bilirubin sample pools (15 mg/dL and 25 mg/dL) with purified digoxin revealed a higher concentration (over recovery) for the DxI, compared to the other two platforms (Fig. 1). The recovery increased with higher concentrations of bilirubin. The Vitros and Cobas showed recoveries closer to the expected concentration for both bilirubin pools, consistent with lack of interference by bilirubin in these two assays. Similarly, results from the mixing study showed the DxI over recovering digoxin (Fig. 1A), while the Vitros and Cobas recovered digoxin closer to the expected concentration (Fig. 1B & 1C).

Fitting parameter results were calculated for the DxI results according to Eq. (2). With estimated standard errors, the fit values were $a = 0.190 \pm 0.005$, $b = 0.806 \pm 0.096$, and $c = 0.478 \pm 0.132$. A plot with the known, DxI 800 reported, and fit corrected digoxin concentrations is presented in Fig. 1A. For a sense of the estimated

Table 2
Inter-instrument comparison of neat, non-icteric plasma samples from patients who were receiving digoxin.

DxI Digoxin (original value)	DxI Digoxin	DxI Absolute bias	DxI Percent recovery	Vitros® Digoxin	Vitros® Absolute bias	Vitros® Percent recovery	Cobas® Digoxin	Cobas® Absolute bias	Cobas® Percent recovery
0.27	0.39	−0.02	95.12%	0.4	0.13	148.15%	< 0.4	*	*
0.36	0.44	0.03	107.32%	< 0.4	*	*	< 0.4	*	*
0.39	0.42	0.03	107.69%	< 0.4	*	*	< 0.4	*	*
0.41	0.73	0.03	104.29%	0.6	0.19	146.34%	< 0.4	*	*
0.41	0.38	0.02	105.56%	0.5	0.09	121.95%	< 0.4	*	*
0.70	0.36	0.09	133.33%	0.6	−0.1	85.71%	0.43	−0.274	60.86%
0.70	0.80	0.1	114.29%	0.8	0.1	114.29%	0.66	−0.043	93.86%
1.66	2.27	0.5	126.82%	1.3	−0.36	78.31%	1.22	−0.440	73.49%
1.79	1.65	−0.01	99.40%	1.9	0.11	106.15%	1.91	0.120	106.70%
3.10	3.01	−0.09	97.10%	3.1	0	100.00%	2.69	−0.410	86.77%

* Unable to calculate.

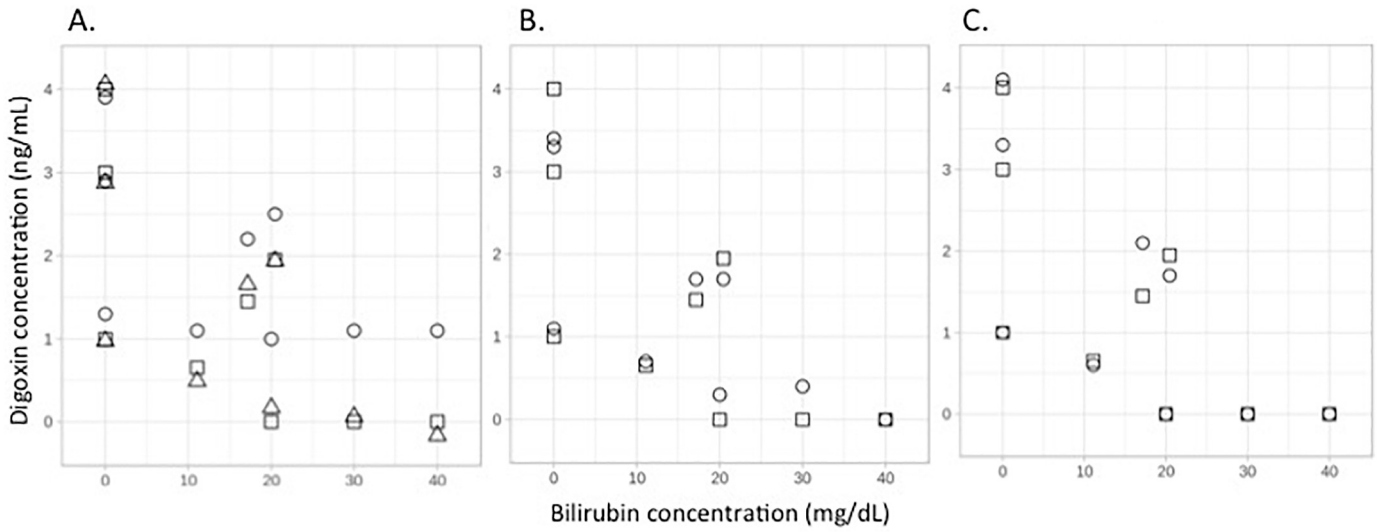

Fig. 1. Comparison of digoxin concentrations from mixing and spiking studies for the DxI (A), the Vitros (B), and the Cobas (C). Expected digoxin concentration is indicated with a square; measured digoxin concentration is indicated with a circle. The corrected DxI 800 values were calculated with the fitting parameters derived using data fitting with Eqs. (1) and (2) and reported values (triangles; pannel A). Samples which gave a result below the limit of quantitation for an assay were plotted as digoxin concentration of zero.

uncertainty in the corrected value (see Eq. (3)), assuming values of [bilirubin] = 20 ± 1 mg/dL, z = DxI reported [digoxin] = 1.0 ± 0.05 ng/mL, and the above fitting parameters, the result is 0.2 ± 0.6 ng/mL. In this case, most of the contribution to the error is contributed by the estimated error in the bilirubin concentration measurement.

4. Discussion

In the presence of high bilirubin, the DxI 800 is the only digoxin assay of the three tested that exhibits clinically significant interference, resulting in a positive bias. Risks for patients include digoxin concentrations being reported as in the therapeutic range when they are actually subtherapeutic, or those in the therapeutic range reported as being in the toxic range. Overall, patients are at risk of being undertreated if clinicians reduce digoxin dosage as a result of testing.

The type of antibody used might explain the differences in bias between the DxI and the other two systems. In the DxI assay, polyclonal rabbit antibodies to digoxin are used, whereas the Vitros and Cobas utilize monoclonal mouse antibodies. Potential interference by bilirubin might include bilirubin prohibiting the binding of the label to the anti-digoxin antibody, causing a decrease in signal (and, consequently, an increased reported digoxin concentration). Another possible explanation may be that bilirubin is directly binding to digoxin, thus preventing the binding of the label to the anti-digoxin antibody, thus falsely elevating detected digoxin in patient plasma. It is unlikely that bilirubin interferes with the label (alkaline phosphatase) since other assays that use this label on the same instrument do not demonstrate interference with high bilirubin concentrations. Other assay differences, as described in the Materials and Methods, could play a role in the difference in bias between the DxI 800 and other assays.

Currently, the University of Washington's procedure for digoxin measurement calls for specimens with total bilirubin > 15 mg/dL simply to be cancelled. As a result of our data, we intend to modify our procedures for these icteric samples. One solution would be to send out testing to laboratories that utilize the Vitros or Cobas assays, which has also been done previously at our institution. Alternatively, if Beckman instrumentation is utilized, evaluating the Digoxin assay on the AU (unfortunately, we did not have access to this assay for evaluation in this study). A final solution would be to use the data modeling approach above to calculate coefficients that can be used to correct the reported DxI digoxin concentrations for bilirubin interference.

While we have focused on bilirubin as an interfering substance, it is helpful for clinicians and laboratorians to be aware of other potential interferences. Digoxin-like immunoreactive factors (commonly found in neonates [9], pregnant women, patients with renal or hepatic failure [11], critically ill or volume expanded patients [12], and also ingested [13,14]) can also potentially interfere with the assay by competitively binding to the detection antibody. Exogenous compounds such as certain drugs (spironolactone, potassium canrenoate, Digibind®) and

Chinese medicines (Chan Su, Lu-Shen Wan, oleander herbal preparations) can also interfere with digoxin immunoassays [13]. Significantly lipemic samples are generally rejected before the assays are performed (e.g., the University of Iowa, using the Cobas® 8000, rejects samples with lipemia > 1500 mg/dL Intralipid); further characterization of lipemia's interference is of interest. Although this study has focused on elevated bilirubin levels, further studies to determine whether a difference between conjugated and unconjugated bilirubin is warranted.

In summary, the spiking and mixing studies demonstrate that increasing concentrations of bilirubin interfere with measurement of digoxin on Beckman Coulter DxI 800 instruments. The digoxin concentration is important to know in many clinical situations wherein dangerous overmedication might be suspected, or the digoxin dosage is insufficient to produce any therapeutic effect. Knowledge of how elevated bilirubin concentrations can interfere with measurement assays can lead to better interpretation of values and use of alternative measurement assays when necessary.

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