

Spurious Hemolysis Does not Influence the Reliability of Digoxin Testing on Siemens RXL MAX and Roche Cobas e601.

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Abstract. Little information is available on the influence of spurious hemolysis on digoxin immunoassays. Seventeen consecutive, non-hemolyzed, sodium-heparin samples were divided in three aliquots. The first was immediately centrifuged and tested for hemolysis index (HI), as well as plasma digoxin on Siemens RXL MAX using the Siemens Dimension Flex and Roche Cobas e601 by electrochemiluminescent (ECLIA) technique. The second and third aliquots were subjected to mechanical hemolysis by aspirating the blood one and two times through a thin needle. The concentration of digoxin measured on Siemens RXL MAX was significantly decreased from aliquot #A, to aliquot #B (-4%), and aliquot #C (-6%), but in none of the hemolyzed specimens the 10% bias was exceeded. No significant variation was observed by measuring plasma digoxin on Roche Cobas.

Key words: hemolysis, hemolyzed specimens, interference, digoxin

Introduction

Digoxin, also known as digitalis, is a purified cardiac glycoside extracted from the foxglove plant *Digitalis lanata*, which is widely used in the treatment of a variety of heart disorders, including atrial fibrillation, atrial flutter, as well as a positive inotrope in heart failure [1]. Laboratory monitoring of digoxin therapy is often indicated for the narrow therapeutic index (e.g., there is only a small difference between therapeutic and toxic drug concentrations), as well as for the potential pharmacokinetics changes associated with several physiological or pathological conditions that might increase its inherent risk of toxicity. These include altered absorption due to slower gut or transdermal absorption, changed bioavailability or biodistribution, altered metabolism and impaired elimination due to slower renal excretion in patients with impaired renal function, increased susceptibility to drug

sensitivity, ageing, co-existing pathologies, polypharmacologic therapy and drug interactions [2]. In most of these circumstances, drug therapy is difficult to predict, so the administration should be started at a low dose and increased slowly under close laboratory monitoring to establish efficacy and prevent toxicity. The drug has a relatively long initial distribution phase of 4 to 8 hours which reflects the distribution from the central compartment to peripheral tissues compartments. So the concentration should not be typically assessed until drug concentrations have reached a steady state, i.e., when the rates of drug absorption and clearance are balanced. Digoxin also displays an average half-life of 38 hours, so that its concentration should be assayed 5-7 days after initiation or modification in dosage [3]. The conventional therapeutic range of plasma digoxin in appropriate laboratory monitoring (i.e., assessment after the pharmacological steady state had been achieved) is 0.9–2.0 ng/mL (1.2–2.6 nmol/L), whereas plasma concentrations >2.0 ng/mL (2.6 nmol/L) are classified as toxic [4].

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Individualized therapy with digoxin thereby requires accurate and reliable therapeutic drug monitoring to safely achieve the desired clinical effects, and to establish a relationship between plasma drug concentration and therapeutic response and/or toxicity. Several types of interference have been previously described for certain digoxin immunoassays, including digoxin-like immunoreactive substances (DLIS) (which are typically increased in newborns, patients with volume expansion, uremia, liver disease, essential hypertension, cardiomyopathy, congestive heart failure, and diabetes) [5], antidigoxin Fab fragments [6], spironolactone, canrenone or potassium canrenoate [7], oleander [8], as well as herbal supplements, complementary and alternative medicines [9-12]. Heterophilic antibodies present in the specimen might also interfere with digoxin immunoassays, complicating therapeutic digoxin monitoring [13]. Despite the fact that these biological and analytical interferences are now clearly recognized as potential sources of bias in digoxin test results, no reliable and recent information is available on the influence of spurious hemolysis, the leading source of variability in laboratory diagnostics [14-16] on commercial digoxin immunoassays.

Materials and Methods

All consecutive, non-hemolyzed, sodium-heparin (Vacuette, Greiner Bio-One GmbH, Frickenhausen, Germany), inpatient samples referred to our laboratory for routine monitoring of plasma digoxin were collected over 3 consecutive working days (n=17; 14 females and 3 males; mean age 82 years [53-93 years]). Three aliquots were immediately obtained from the primary tube upon arrival in the laboratory. The first (“#A”) was immediately centrifuged at 2000xg for 15 min at room temperature. Plasma was then tested for the hemolysis index (HI) on a Beckman Coulter DxC 800 (Beckman Coulter Inc., Brea CA, USA). Plasma digoxin was assessed with two different immunoassays on a Siemens RXL MAX[®] and Roche Cobas e601. The second (“#B”) and third (“#C”) aliquots were subjected to mechanical hemolysis by aspirating the heparinized blood one (aliquot “#B”) and two times (aliquot “#C”) with a 0.5 mL insulin syringe equipped with a very thin needle (30 gauge, 0.3x8 mm)

(Picindolor, Artsana S.p.A., Grandate, Italy). This method closely mirrors a traumatic blood collection, causing concomitant injury to erythrocytes, platelets and leukocytes [17]. The plasma was then separated by centrifugation at 1500xg for 10 min at room temperature and tested for HI and plasma digoxin with both immunoassays. The HI has been assessed on a Beckman Coulter DxC by direct spectrophotometry, a technique that significantly correlates with the reference cyanmethemoglobin assay [18].

The Siemens Dimension[®] Flex[®] method is an immunoassay technique for the quantitative measurement of digoxin in serum and plasma on Siemens RXL MAX[®] platform, in which free and digoxin-bound antibody-enzyme species are separated using magnetic particles. The methodology involves antibody conjugate reagent mixing with patient's serum or plasma. The antibody conjugate reagent utilizes the F(ab)₂ fragment of the antibody to eliminate interference from rheumatoid factor. Digoxin in the sample is bound by the F(ab)₂-β-galactosidase (β-gal) in the antibody conjugate reagent. Magnetic particles coated with the digoxin analog ouabain are added to bind free (unbound) antibody-enzyme conjugates. The reaction mixture is then separated magnetically, and the supernatant containing the digoxin-antibody-enzyme complex is transferred and mixed with a substrate. The B-gal portion of the Digoxin-F(ab)₂ β-gal complex catalyzes the hydrolysis of chlorophenol-β-D-galactopyranoside (CPRG) to chlorophenol red (CPR). The change in absorbance at 577 nm due to the formation of CPR is directly proportional to B-galactosidase activity. Since β-gal is not present in serum, its activity is directly proportional to digoxin in the patient's sample and is measured using a bichromatic (577, 700 nm) rate technique. The analytical measurement range of this assay is 0.06-5.00 ng/mL, the total imprecision <5.2%. The digoxin is measured in Roche Cobas e601 by a two step sandwich immunoassay with streptavidin microparticles and is a electrochemiluminescent (ECLIA) detection. Digoxin in the specimen competes with the added digoxin derivative labeled with biotin for the binding sites on the ruthenylated antibody-complex. Streptavidin-coated microparticles are added and the mixture is aspirated

into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Application of voltage to the electrode induces the chemiluminescent emission, which is then measured. The analytical measurement range of this assay is 0.15-5.00 ng/mL, the total imprecision <7.4%.

Since the variables were not found to be normally distributed by Kolmogorov-Smirnov test, results were finally expressed as a geometric mean and 95% Confidence of Interval (95% CI). The non-parametric Wilcoxon matched pairs test was used for statistical analysis. A bias $\geq 10\%$ from the reference sample with no hemoglobin interference was considered as a significant variation [19]. The study was carried out in accordance with the Declaration of Helsinki and under the terms of all relevant local legislation.

Results

The results of this investigation are shown in **figure 1**. A negligible amount of cell-free hemoglobin was present in aliquot #A (cell-free hemoglobin <0.5 g/L), whereas a gradually increasing value was present in aliquots #B (cell-free hemoglobin between 14.5 and 27.5 g/L) and #C (cell-free hemoglobin between 30.5 and 45.0 g/L). The concentration of plasma digoxin measured on Siemens RXL MAX[®] was significantly decreased from aliquot #A (0.92 ng/mL, 95% CI 0.54-2.09 ng/mL) to aliquot #B (0.88 ng/mL, 95% CI 0.52-2.08 ng/mL; $p < 0.001$), and further to aliquot #C (0.86 ng/mL, 95% CI 0.51-2.04 ng/mL; $p < 0.001$) (**Figure 1A**). The median decrease of plasma digoxin with this assay was 4% (95% CI 0-7%) in aliquot #B and 6% (95% CI 1-9%) in aliquot #C, respectively. The 10% variation from the non-hemolyzed specimen was not exceeded in any of the sample aliquots #B and #C. No analytically or clinically significant variation was instead observed by measuring plasma digoxin on Roche

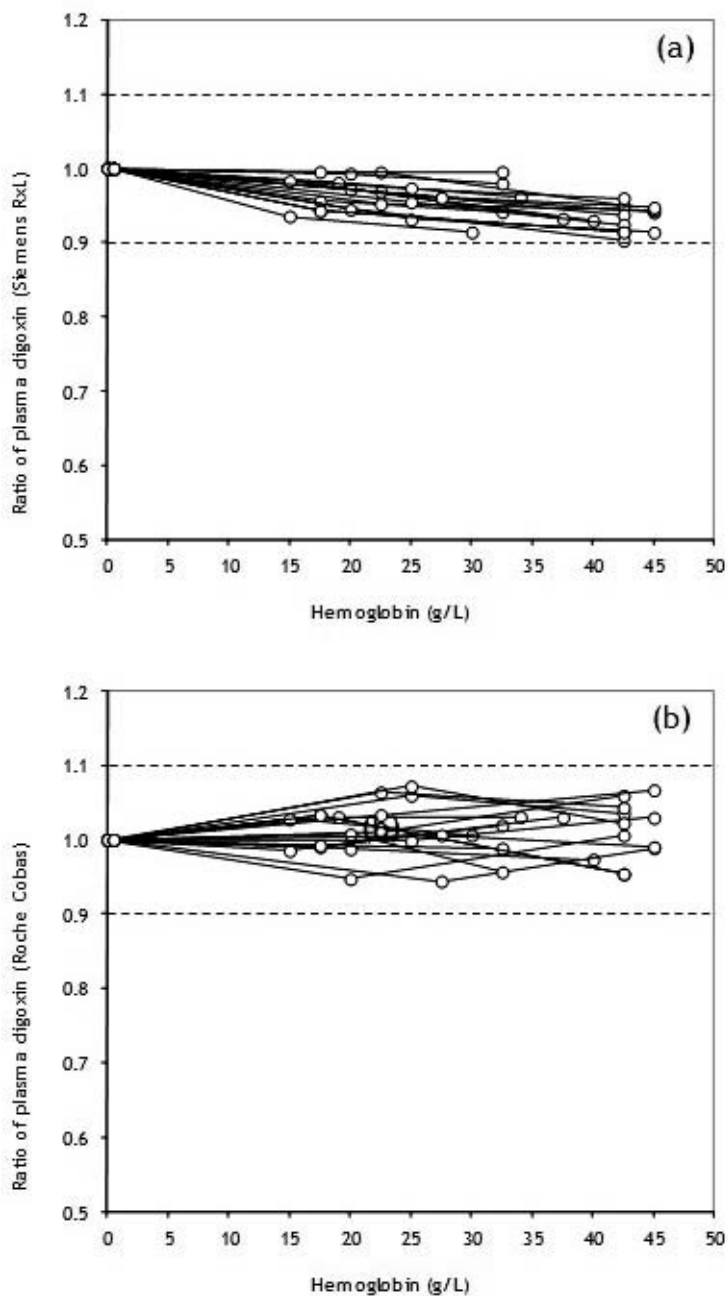


Figure 1. Ratio of plasma digoxin concentration between non-hemolyzed and hemolyzed specimens, as measured on Siemens RXL MAX (a) and Roche Cobas e601 (b). The dotted line demarcates a 10% variation of plasma digoxin values from the non-hemolyzed specimen.

Cobas (**Figure 1B**). The concentration was in fact 0.91 ng/mL (95% CI 0.53-2.36 ng/mL) in aliquot #A, 0.92 ng/mL (95% CI, 0.56-2.36 ng/mL; $p = 0.40$) in aliquot #B and 0.92 ng/mL (95% CI, 0.55-2.39 ng/mL; $p = 0.1$) in aliquot #C. The 10% variation from the non-hemolyzed specimen was not exceeded in the sample aliquots #B and #C.

Discussion

Hemolytic specimens are the leading cause of pre-analytical variability in laboratory diagnostics and are a recognized source of problems for both laboratory professionals and clinicians [15,16]. The test results of several analytes are in fact biased and thereby unreliable in frankly hemolyzed samples. So their necessary suppression delays the triage of patients, as well as the clinical and therapeutic decision making, at least until an additional, suitable specimen is received and analyzed [14,15].

The monitoring of plasma digoxin concentration is advisable for a variety of clinical circumstances and requires a simple, quick, and reliable analysis due to the important therapeutic implications. Although there is comprehensive information on a variety of biological and analytical sources of interference in digoxin immunoassays, data on the potential bias caused by spurious hemolysis is scarce and even controversial. Moreover, most of the published studies have assessed old methods, which are no longer available in the market. Lehmann observed a positive bias on SYVA Advance Digoxin assay in sera containing hemoglobin at concentrations >1.0 g/L, which occurred despite the method used a pretreatment reagent for minimizing this type of interference [20]. No significant interference from hemolysis was observed in other investigations using the Abbott TDx fluorescence polarization immunoassay [21,22]. More recently, Ma et al showed no interference from up to 50 g/L of hemoglobin-based oxygen-carrying (HBOC) solution on the Abbott AxSym digoxin immunoassay [23].

Although manufacturer's datasheets report no interference from cell-free hemoglobin up to 10 g/L for both Siemens Dimension® Flex® and Roche Cobas e601, no definitive evidence has been provided to confirm these claims for the former assay to the best of our knowledge, whereas Ji and Meng recently reported that digoxin concentration might be significantly decreased on Roche Cobas 6000 in the presence of 1.0 g/L of cell-free hemoglobin [24]. The results of our investigation attest that despite the different technical features (i.e., Flex Reagent Cartridge versus ECLIA technique), these two digoxin immunoassays are extremely robust

against hemolysis, so test results can be reliably reported to the clinicians even in the presence of gross hemolysis up to 45 g/L. This is not surprising inasmuch as both technologies are supposed to be very robust against interference as compared with previous immunoassay that are not based upon separation of the immune-complex from the plasma. The Flex Reagent Cartridge uses in fact particles that are completely (magnetically) separated from the interfering substances potentially contained in the supernatant, whereas the ECLIA technique uses streptavidin-coated microparticles that are also mechanically separated from the supernatant and the potentially interfering substances. In both cases, however, the cell-free hemoglobin potentially present in the sample is eliminated from the final reaction buffer.

In conclusion, since the vast majority of spuriously hemolyzed specimens detected in the daily laboratory practice are characterized by cell-free hemoglobin concentration below 1 g/L [14,15], hemolysis should not be thereby considered a major limitation in routine digoxin testing with Flex Reagent Cartridge and ECLIA techniques.

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