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Short Communication

Factors influencing naproxen metabolite interference in total bilirubin assays

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ABSTRACT

Background: The factors influencing naproxen metabolite O-desmethylnaproxen (ODMN) positive interference in diazo-based Jendrassik and Grof (JG) total bilirubin (Tbil) assays and lack of interference in direct bilirubin (Dbil) assays have not been resolved. The objective of this study was to understand the conditions causing this interference pattern.

Methods: Pooled normal and ultra-filtered plasma samples spiked with ODMN and naproxen were measured on the Beckman Coulter DxC and AU instruments. Absorbance spectra were obtained for ODMN mixed with Dbil reagent at original and adjusted pH. Absorbance spectra were also obtained for ODMN and bilirubin samples mixed with Tbil assay reagents.

Results: ODMN produces a positive interference in the DxC JG Tbil assays, but not the AU Tbil or Dbil assays or the DxC Dbil assay. Neutralizing the acidic pH of AU and DxC Dbil reagents allows ODMN to react with diazo salts. ODMN samples mixed with DxC and AU Tbil reagents produce broad peaks from 450 to 560 nm and 400 to 540 nm, respectively. The DxC JG Tbil assay monitors a change in absorbance at 520 nm close to peak absorbance wavelength of diazo-reacted ODMN, whereas the AU Tbil assay monitors a change in absorbance at 570/660 nm, beyond the peak absorbance wavelengths of diazo-reacted ODMN.

Conclusion: The acidic pH of diazo-based Dbil assay reagents inhibits the reaction of ODMN with diazo salts. The AU JG Tbil assay is a reliable method to measure Tbil in the setting of naproxen overdose.

1. Introduction

Bilirubin is a heme degradation product often monitored in the clinical evaluation of drug toxicity or overdose [1]. We were recently alerted to a case of spurious hyperbilirubinemia in a 57-year old woman who overdosed on two-thirds of a bottle of naproxen. Her labs were significant for markedly elevated total bilirubin 164.2 umol/L or 9.6 mg/dL, reference interval 3.2-22.2 umol/L or 0.2-1.3 mg/dL, but normal direct bilirubin at 1.7 umol/L or 0.1 mg/dL (0.0-5.1 umol/L or 0.0-0.3 mg/dL). This suspicious pattern of isolated elevated total bilirubin without jaundice or other signs of liver disease led our clinicians to suspect an interference with the total bilirubin assay run on our institution's chemistry analyzer.

Naproxen [sodium (2S)-2-(6-methoxynaphtalen-2-yl)proponoate] is a non-steroidal anti-inflammatory drug (NSAID) commonly used for relief of pain and inflammation. Demethylation of naproxen produces the major naproxen metabolite, O-desmethylnaproxen (ODMN). Both naproxen and ODMN are glucoronidated before excretion into the urine and feces (95% and 5%, respectively) [2].

Therapeutic use of naproxen does not lead to any known interference with bilirubin assays. In contrast, when excess naproxen is ingested, the naproxen metabolite ODMN (but not the methylated parent drug naproxen) has been found to interfere with traditional Jendrassik and Grof (JG) diazo-based total bilirubin (Tbil) assays on several automated chemistry analyzers including the Siemens (Bayer) Advia 1650, Dade Behring RXL-Max, Beckman Coulter Synchron DxC 800, and Siemens Dimension Vista [3–6]. However, not all bilirubin assays are affected. For example, a previous study found that the Roche Tbil assay shows no interference with high concentrations of ODMN [6]. Yet, it is unclear why ODMN produces a positive interference in some but not all diazo-based bilirubin assays.

Previous studies also showed that direct bilirubin (Dbil) measurements are unaffected by high concentrations of ODMN and hypothesized that the caffeine accelerator in the JG Tbil method dissociates ODMN from albumin and accelerates the reaction of ODMN with the diazo reagent [5,6]. The objective of this study was to understand the conditions that promote positive interference with ODMN in the affected bilirubin assays.

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2. Materials and methods

2.1. Reagents/materials

Naproxen sodium (N5160), ODMN (UC305), dimethyl sulfoxide (DMSO, D2650), bilirubin powder (B4126), and 3 M sodium acetate (S-7899) were purchased from Sigma Aldrich (St. Louis, MO) and stored at room temperature. Stock solutions of the following were prepared: 20 mM naproxen in DMSO (stored at room temperature), 20 mM ODMN in DMSO (stored at room temperature), and 10 mg/L bilirubin (made fresh in 0.1 N NaOH and protected from light).

DxC Tbil reagent (REF 476861, Lot M407248), DxC Dbil reagent (REF 476856, Lot M404327), AU Tbil reagent (REF OSR6112, Lot 6922), and AU Dbil reagent (REF OSR 6111 Lot, 6841) were obtained from Beckman Coulter (Brea, CA).

Ultra-filtered pooled plasma (FPP) was prepared by centrifuging pooled normal patient plasma in Millipore Centrifree Centrifugal Filter Units (Millipore, Cat. #4104) at 22–28 °C in a fixed-head refrigerated centrifuge (Eppendorf Centrifuge 5702R #40) at 3400 rpm for 18 min. Albumin and microalbumin assays run on the DxC were used to confirm undetectable protein concentration in FPP.

2.2. Instruments/assays

All bilirubin assays were performed on the Beckman Coulter Synchron DxC 800 or AU 680 analyzers (Brea, CA) according to manufacturer's instructions.

The DxC Dbil assay reagent consists of 27 mM sulfanilic acid, 51 mM HCl, and 0.12 mM sodium nitrite, which react to form diazotized sulfanilic acid. The diazotized sulfanilic acid combines with conjugated bilirubin to form a colored compound, which is quantified by monitoring endpoint absorbance at 560 nm. The coefficient of variation (CV) for the DxC Dbil assay was 1.5% at 98.8 μmol/L (5.78 mg/dL) and 12.6% at 5.0 μmol/L (0.29 mg/dL). In the DxC Tbil method, both conjugated and unconjugated bilirubin react with the diazotized sulfanilic acid in the presence of a caffeine reagent (173.9 mM caffeine, 347 mM sodium benzoate, 203 mM sodium acetate) and formation of a colored compound is monitored with endpoint absorbance at 520 nm. The CV of the DxC Tbil assay was 2.1% at 287 μmol/L (16.8 mg/dL) and 17.3% at 15.4 μmol/L (0.9 mg/dL).

The AU Dbil and Tbil assays are similar to the DxC bilirubin assays, but utilize a stable diazo salt (3,5-dichlorophenyldiazonium tetrafluoroborate) and an endpoint bichromatic method that monitors absorbance at 570 (primary wavelength) and 660 nm (secondary wavelength). The AU Tbil reagent contains surfactant in addition to the stable diazo salt and caffeine. The CV of the AU Dbil assay was 3% at 42.8 μmol/L (2.5 mg/dL) and 16.2% at 5.1 μmol/L (0.3 mg/dL), and the CV of the AU Tbil assay was 1.1% at 118.8 μmol/L (6.95 mg/dL) and 2.3% at 12.0 μmol/L (0.7 mg/dL).

The reagent pH measurements were performed on the Corning Model 440 pH Meter.

The HP 8453 UV–Vis Diode Array System was used to capture the complete ultraviolet to visible light spectra (200–1100 nm) of the bilirubin assay reagents mixed with bilirubin and ODMN.

2.3. Interference assessed using the DxC and AU automated chemistry analyzers

Naproxen and ODMN were added to normal pooled plasma (NPP) and ultra-filtered pooled plasma (FPP) (final concentrations of 1 mM naproxen, 4 mM naproxen, 0.5 mM ODMN, or 1 mM ODMN) and analyzed for total and direct bilirubin using the AU and DxC assays. These samples were analyzed once on the AU and in duplicate on the DxC.

Bilirubin and ODMN (final concentrations 171 μmol/L (10 mg/dL) and 1 mM, respectively) were added to a second preparation of NPP, and the Dbil and Tbil were quantified with single measurements on both the DxC and AU analyzers.

2.4. Spectral characterization of ODMN interference

DxC and AU reagent components were mixed at ratios directed by the manufacturer's instructions and as completed by the analyzers. ODMN (1 mM in water) or an icteric plasma sample (412.1 μmol/L (24.1 mg/dL) Tbil and 191.5 μmol/L (11.2 mg/dL) Dbil) were added to the reagent mixtures at the same sampling ratio for each individual instrument. In control samples, water was added to correct for any dilution effects. For pH interference studies with Dbil reagents, sodium acetate and sodium hydroxide were used to adjust DxC and AU Dbil reagent pH to 5.5 and 5, respectively. Spectra of all samples were obtained using the HP 8453 UV–Vis Diode Array System.

3. Results

3.1. Interference in normal and bilirubin-spiked pooled plasma

The naproxen metabolite ODMN is responsible for the positive interference observed in Tbil quantification on the DxC analyzers as demonstrated by spiking normal pooled plasma (NPP) with ODMN (Table 1). In contrast, the DxC Dbil assay showed no interference with up to 1 mM ODMN in NPP. NPP with up to 4 mM naproxen also did not show any interference in the DxC Dbil or Tbil assays. Interestingly, NPP or FPP spiked with up to 4 mM naproxen or 1 mM ODMN showed no interference in the AU Dbil or Tbil assays (Table 1).

The effect of ODMN in icteric samples was also evaluated (Table 1). NPP samples with 171 μmol/L (10 mg/dL) conjugated bilirubin and 1 mM ODMN showed accurate Tbil quantification on the AU analyzer (164.2 μmol/L; 9.6 mg/dL), but not the DxC analyzer (343.7 μmol/L; 20.1 mg/dL). Thus, even in icteric samples, ODMN produces a positive interference on the DxC, but not the AU assay.

3.2. Interference in ultra-filtered plasma

A previous study speculated that ODMN does not interfere in the diazo-based Dbil assays because like unconjugated bilirubin, ODMN is largely protein-bound and is therefore sterically hindered from reacting with the diazo reagent [3]. However, up to 1 mM ODMN spiked into ultra-filtered pooled plasma (FPP) with undetectable (<2 g/L) albumin did not demonstrate any positive interference in the DxC or AU Dbil assays (Table 1). This lack of ODMN interference in the Dbil assays with FPP suggests that this previous hypothesis was incorrect.

3.3. Effect of pH on ODMN-diazo reaction spectra

Reagent pH of the direct and total bilirubin assays differ considerably. Both the DxC and AU Dbil assay reagents have low pH values of 1.5 and <1, respectively, while the AU and DxC Tbil assay reagents have pHs of 4.6 and 6.7, respectively. To evaluate whether increasing the pH of the Dbil assay reagents would allow reaction with ODMN, Dbil reagents at the original reagent pH and at an adjusted pH (5.5 for DxC Dbil and 5 for AU Dbil) were mixed with a 1 mM ODMN solution at the sampling ratio specific to the DxC or AU. Absorbance spectra of ODMN mixed with DxC and AU Dbil reagents at their original acidic pH show minimal change from baseline reagent spectra; however, ODMN mixed with Dbil reagents at adjusted pH results in absorbance with peaks from 440 to 560 nm for DxC Dbil reagent at pH 5.5 (Fig. 1A) and 400–600 nm for AU Dbil reagent at pH 5 (Fig. 1B). These experiments show that ODMN does not produce any color change in the acidic pH of the DxC and AU diazo-based Dbil reagents.

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