Elucidation of a Naproxen Metabolite Interference in Total Bilirubin Testing on a Routine Chemistry Analyzer System Using LC-MS/MS

Laboratory Medicine and Pathology

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Abstract

The CYP450-based demethylation of the anti-inflammatory drug naproxen leads to a metabolite – *o*-desmethylnaproxen – that rapidly reacts with the sulfanilic acid-based chemistry in the classic Jendrassik & Gròf method for total bilirubin. This chemistry is currently used in a significant number of automated clinical analyzer systems. Here we found experimental mass spectrometric along with spectroscopic data that is consistent with formation of a novel substituted naphthalene ring-based azo dye related to the Orange G histochemical stain. This compound is likely responsible for the positive test interference seen in patients with markedly elevated serum naproxen levels when using chemistry platforms employing such reagents.

Introduction

Measurement of total bilirubin in the clinical chemistry lab is typically done via chromogenic test modalities. These tests are well suited for rapid, high-volume testing situations on automated analyzer platforms. The tests are robust and interfering substances are generally well characterized though exceptions exist – particularly for drug metabolites.

Naproxen is a nonsteroidal anti-inflammatory drug (NSAID) widely used clinically for both analgesia and for its anti-inflammatory properties. Naproxen is a stereochemically pure compound in the 2-arylpropionic acid class. Absorption of naproxen is rapid and complete when given orally.[1] Hepatic metabolism of naproxen includes cytochrome P450-based *o*-demethylation of the napthalenic methoxyl group to form *o*-desmethylnaproxen (ODMN) – primarily through the 2C9 isoform with lower contributions from the 1A2 and 2C8 isoforms.[2,3] Whether further processing of ODMN occurs in the kidney proper remains unclear.[4] Notably, the kinetics of naproxen can be altered in hepatic disease and rheumatoid arthritis.

Excretion of the conjugated *o*-desmethylnaproxen metabolite may be connected to a patient's renal function: end-stage renal disease leads to accumulation of ODMN but does not appear to be specifically altered by age.[1]

In the original Jendrassik & Gròf total bilirubin assay (1938)[5], conjugated and unconjugated bilirubin measurements are obtained simultaneously via reaction with a diazo dye. Diazotized sulfanilic acid reacts directly with conjugated bilirubin, while the caffeine and benzoate "accelerator" is predicted to drive the release of unconjugated bilirubin from albumin, thus allowing the unconjugated form to react directly with the diazo compound and forming azobilirubin.

This colorimetric method is generally robust, unaffected by wide pH changes, and insensitive to high levels of protein, while remaining spectrophotometrically sensitive at low, physiologically-relevant bilirubin ranges. The Jendrassik & Gròf method forms the foundation of many routine bilirubin measurements performed in clinical labs on automated analyzer systems. Recently it has been shown empirically that this classic analytical method is susceptible to strong positive interference by the ODMN metabolite in patients with markedly elevated serum naproxen levels, leading to erroneously elevated total bilirubin reports.[6,7]

Here we use spectrophotometric and LC-MS/MS analytical techniques to demonstrate that a metabolic product of naproxen metabolism (*o*-desmethylnaproxen) reacts with the diazo reactant, producing a bright orange dye structurally similar to the classic histochemical stain acid orange 7 (Orange G/Orange II).

Experimental

All non-test kit chemicals and chromatography reagents were purchased from Sigma or Fisher. Physiologic artificial bilirubin samples with and without naproxen and/or ODMN were processed as specified by the test manufacturer (Siemens [TBIL] or Roche/Cobas [BILT.3]) for on-instrument testing; off-instrument testing closely replicated the on-instrument conditions.

UV-Vis spectroscopy: Samples were read over a 400-600 nm range at 0.2 nm resolution using a quartz cuvette on a UV-1600PC spectrophotometrer (VWR); all measurements were done in triplicate.

Mass spectrometry: Samples were run on an XR HPLC system (Shimadzu Scientific) connected to a QTRAP 6500 mass spectrometer (AB SCIEX) using electrospray ionization in negative ionization mode. Chromatography was performed using a Synergi 4 µm Fusion-RP 80 Å, 50 x 2 mm column (Phenomenex) and with a 3.5 min linear-gradient using water (mobile phase A) and acetonitrile (mobile phase B), both with 10 mM ammonium formate.

MRMs were individually constructed for naproxen (229.09-185.0 and 229.09-170.0), ODMN (215.08-169.0 and 215.08-171.1) and the predicted diazo product (399.07-355.0, 399.07-170.9 and 399.07-155.8).

Results

As previously reported[6], the Siemens TBIL reaction is dramatically affected by the presence of the naproxen metabolite ODMN, resulting in a marked shift in the absorption spectrum around the 540 nm reading point (fig 1, 2); this shift is not observed with elevated levels of the parent compound naproxen. We confirmed this spectrophotometric anomaly for the Siemens TBIL testing system and investigated this for the new (2014) Roche/Cobas BILT.3 reagents: this new formulation uses a different dye chemistry and the Roche/Cobas system is now unaffected by elevated ODMN levels.

Production of a novel diazo product was hypothesized (fig 3). LS-MS/MS of the Siemens BILT test products demonstrated the decrease of the ODMN precursor and the appearance of a new peak consistent with the predicted product, (S)-2-(6-hydroxy-5-((4-sulfophenyl)-diazenyl)naphthalen-2-yl)propanoic acid (fig 4). Post-reaction sample spiking with excess ODMN restored the precursor peak; control samples lacking either ODMN or the diazo reactant failed to produce the predicted product. The results were unchanged whether the reaction was performed in charcoal-stripped serum or non-serum test matrix.

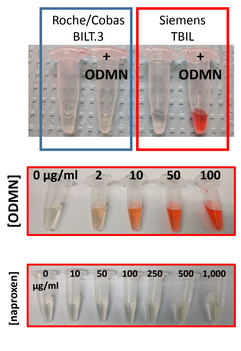
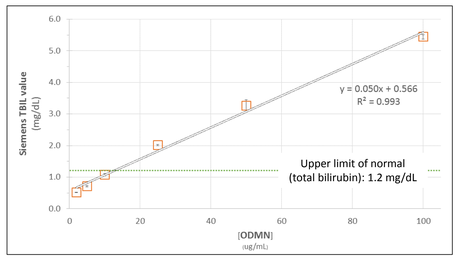


Figure 1a: The addition of ODMN yields a bright orange product with the Siemens TBIL assay (right) , but not the Roche/Cobas BILT.3 (left).

Figure 1b: Increasing [ODMN] yields increasing orange dye product (Siemens TBIL) when tested off-instrument.

Figure 1c: Increasing concentrations of the parent naproxen compound does not affect the TBIL assay.

Figure 1d: On-instrument testing of the TBIL reagents on the Siemens Vista 2500 also shows elevation of the total bilirubin with ODMN addition but not the naproxen parent compound (not shown).



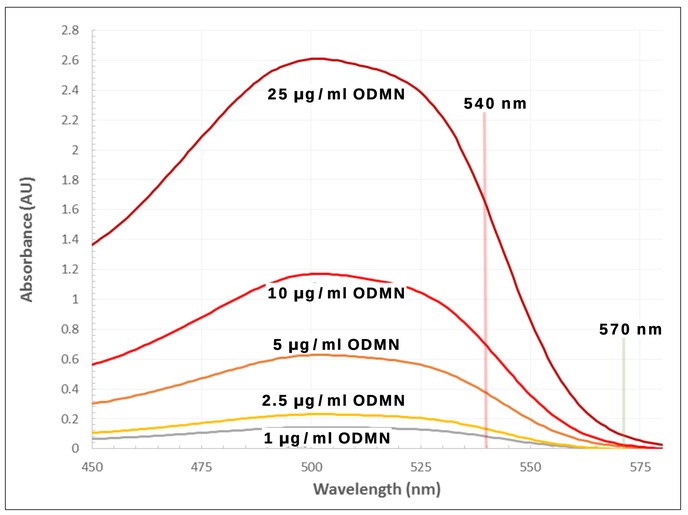


Figure 2: UV-Vis spectroscopy of ODMN spiked samples after off-instrument reaction using the Siemens TBIL assay. Note the rapid rise in absorbance at 540 nm (the one-instrument primary wavelength for this assay) with increasing ODMN concentration.

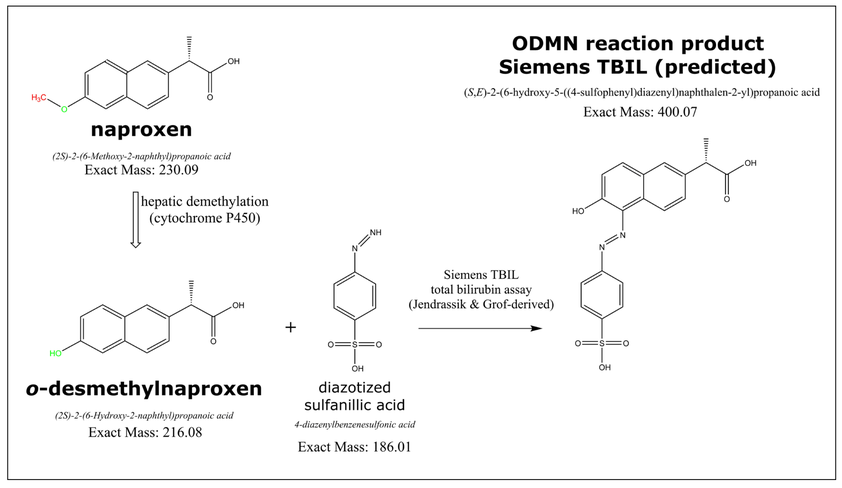


Figure 3: Molecular structures, predicted exact masses, and proposed reaction product of OMDN post-Jendrassik and Grof assay (Siemens TBIL)

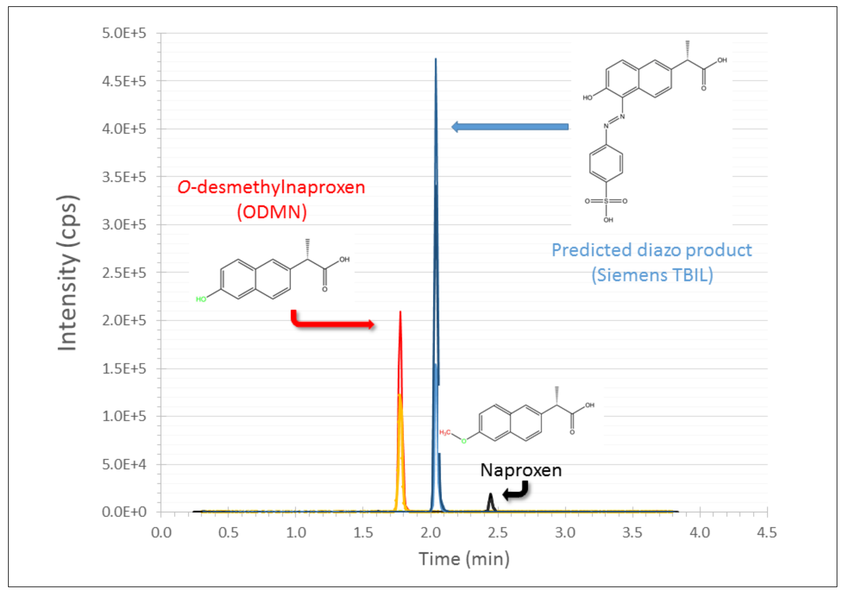


Figure 4: Intensity plots of the post-reaction Siemens TBIL product spiked with ODMN (red arrow), the predicted diazo product (blue arrow) and trace residual naproxen (black arrow

Conclusions / Future Directions

Using LC-MS/MS and spectrophotometric techniques, we have demonstrated that a primary metabolic product of naproxen (*o*-desmethylnaproxen) reacts with the diazo reactant present in the classic Jendrassik & Gròf method for total bilirubin analysis and produces a bright orange dye suggesting this as the cause for spectrophotometric interference previously reported by others.

While some patients appear to produce elevated levels of ODMN on normal naproxen dosing[6], a much larger pool of patients with potentially erroneous TBIL levels tested with affected instrumentation are those with autoimmunue diseases (including arthritis): these patients often take 3-5× the normal dose. Further characterization and confirmation studies of clinical effects via bioinformatic assessment and direct testing of patient serum samples are in progress to determine the potential magnitude of the testing issue on a population level.

References/Acknowledgements

1. Davies, N. M. & Anderson, K. E. Clinical Pharmacokinetics of Naproxen. Clin. Pharmacokinet. 32, 268–293 (1997).
2. Tracy, T. S., Marra, C., Wrighton, S. A., Gonzalez, F. J. & Korzekwa, K. R. Involvement of multiple cytochrome P450 isoforms in naproxen *O*-demethylation. Eur. J. Clin. Pharmacol. 52, 293–298 (1997).
3. Miners, J. O., Coulter, S., Tukey, R. H., Veronese, M. E. & Birkett, D. J. Cytochromes P450, 1A2, and 2C9 are responsible for the human hepatic *O*-demethylation of R- and S-naproxen. Biochem. Pharmacol. 51, 1003–1008 (1996).
4. Vree, T. B., Hekster, Y. A. & Anderson, P. G. Contribution of the Human Kidney to the Metabolic Clearance of Drugs. Ann. Pharmacother. 26, 1421–1428 (1992).
5. Jendrassik, L. & Gróf, P. Vereinfachte photometrische Methoden zur Bestimmung des Blutbilirubins (Simplified photometric methods for the determination of blood bilirubin). Biochem Zeitschrift 297, 82–89 (1938).
6. Dasgupta, A., Langman, L. J., Johnson, M. & Chow, L. Naproxen Metabolites Interfere With Certain Bilirubin Assays. Am. J. Clin. Pathol. 133, 878–883 (2010).
7. Saifee, N. H., Ranjitkar, P. & Greene, D. N. Factors influencing naproxen metabolite interference in total bilirubin assays. Clin. Biochem. 49, 514–517 (2016).

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