PSEUDO-HYPERBILIRUBINEMIA

Hospitalist Conference
8/28/17
Dang Duong
Nomenclature

- Pseudo-hyperbilirubinemia
- Apparent hyperbilirubinemia
- Artifactual hyperbilirubinemia
- Anicteric hyperbilirubinemia
Case:

- 44 yo male with mild diabetes on Metformin presented with mild chest pain. He was ruled out with negative cardiac enzymes and ECG. He appears quite well in ER and could have been discharged home but he was incidentally found to have hyperbilirubinemia of 25 (direct bilirubin 0.1) and was admitted. He was not jaundiced and had no abdominal pain.
Methods of Bilirubin Estimation

- Diazo method
- DPD
- HPLC
- Capillary electrophoresis methods
- Enzymatic methods
- Direct spectrophotometry
- Transcutaneous measurement of bilirubin (non-invasive)
December 5, 1885] THE MEDICAL RECORD.

Reports of Societies.

PRACTITIONERS’ SOCIETY OF NEW YORK.
Stated Meeting, November 6, 1885.
Beverley Robinson, M.D., President, in the Chair.

Dr. C. I. Dana described

A new urinary reaction and test,
first suggested by Professor Ehrlich, of Berlin, as of value in the diagnosis and prognosis of certain febrile diseases.

In 1882 Professor Ehrlich (in Zeitschrift für klinische Medicin, 1882; Charité Annalen, 1883) published his experiments with the diazor-compounds as tests for aromatic bodies in the urine. When aniline (C₆H₅N), which is a nitrogenous body resembling an alkaloid, is mixed with nitrous acid, a series of peculiar bodies result known as the “diazor-compounds.” These compounds have the property of uniting with a large series of bodies, chiefly of the aromatic group, forming colored compounds. Ehrlich prepared a saturated solution of sulfanilic acid in an acidulated solution of water. To this he added a little nitrous acid. The result was the production of a mixture containing diazor-compounds.

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[...]
Azo compound

\[
\begin{array}{c}
\text{R} \\
\text{N=NN} \\
\text{R'}
\end{array}
\]

Diazo compound

\[
\begin{array}{c}
\text{R} \\
\text{C-NN} \\
\leftarrow \\
\text{C-NN}
\end{array}
\]

Azo dyes
van den Bergh method
Diazoo method
Bilirubin

3,5-Dichlorophenyldiazonium tetrafluoroborate

Azobilirubin

Bilirubin + DPD → Azobilirubin

Surfactant
Bilirubin structure
Intramolecular hydrogen bonds in unconjugated bilirubin
Bilirubin conjugation

Conjugation of bilirubin

Unconjugated

COOH  COOH

Conjugated (Water soluble)

COOR  COOR

UDP-glucuronyl transferase

Glucuronic acid
Intramolecular hydrogen bonds disrupted in **conjugated** bilirubin
Another method of bilirubin measurement

Alkaline methanolysis —

Bilirubin \[\xrightarrow{\text{alkaline methanolysis}}\] chloroform extraction \[\xrightarrow{\text{bilirubin methyl esters.}}\]

Separation of these esters is performed using high-performance liquid chromatography (HPLC), and spectrophotometry.
HPLC Based Fractions of Bilirubin

- Alpha (α-bilirubin)
- Beta bilirubin (β-bilirubin)
- Gamma (γ-bilirubin)
- δ (delta) -bilirubin

Classification of Bilirubin based on Diazo Reaction

**Direct Bilirubin:**
- Beta (monocojugated)
- Gamma (diconjugated)
- Delta bilirubin

**Indirect Bilirubin**
- Alpha (unconjugated) bilirubin
Beckman Coulter AU analyzer
SYNCHRON SYSTEM

INTERFERENCES

1. The following substances were tested for interference with this methodology:

Table 5 Interferences

<table>
<thead>
<tr>
<th>SUBSTANCE</th>
<th>SOURCE</th>
<th>MAXIMUM LEVEL TESTED</th>
<th>OBSERVED EFFECT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin</td>
<td>RBC hemolysate</td>
<td>100 mg/dL</td>
<td>≤+0.24 mg/dL</td>
</tr>
<tr>
<td>Lipemia</td>
<td>Intralipid</td>
<td>200 mg/dL</td>
<td>≤-0.24 mg/dL</td>
</tr>
<tr>
<td>Azide</td>
<td>NA</td>
<td>5 mg/dL</td>
<td>≤+0.24 mg/dL</td>
</tr>
<tr>
<td>Citrate</td>
<td>NA</td>
<td>900 mg/dL</td>
<td>≤±0.20 mg/dL</td>
</tr>
<tr>
<td>Oxalate</td>
<td>NA</td>
<td>1000 mg/dL</td>
<td>≤±0.20 mg/dL</td>
</tr>
<tr>
<td>Gentisic Acid</td>
<td>NA</td>
<td>5 mg/dL</td>
<td>≤+0.24 mg/dL</td>
</tr>
<tr>
<td>Acetoacetate</td>
<td>NA</td>
<td>0.2 mg/mL</td>
<td>≤+0.7 mg/dL</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.08 mg/mL</td>
<td>≤+3.7 mg/dL</td>
</tr>
</tbody>
</table>

2. Lipemic samples >2+ should be ultra-centrifuged and the analysis performed on the infranate.

3. The Naproxen metabolite, O-desmethylnaproxen, has demonstrated a positive interference with the Jendrassik-Grof method for total Bilirubin measurement.\footnote{11}

4. Refer to References (12,13,14) for other interferences caused by drugs, disease and preanalytical variables.
Mechanism of Interferences of a lipemic sample

- Light scattering effects may increase absorbances.
- Volume displacement effect greatly decreases the value of some analytes particularly electrolytes; Na+, K+.
- Hemolysis of RBCs is enhanced in the presence of lipemia.
- Non homogeneity of the sample.
- Physical and chemical interferences.
**Relative size of lipid particles**

**Figure 1.** Lipoprotein particle sizes and lipemia. Lipoproteins that are coloured dark grey cause turbidity of the sample. VLDL – very low density lipoproteins (L – large; M – medium; S – small), LDL - low density lipoproteins; HDL - high density lipoproteins.
Wavelengths

- Bilirubin: 540 nm
- Lipid particle: 300 – 700 nm
## Level of lipemia

<table>
<thead>
<tr>
<th>Flag</th>
<th>LIP (mg/dL Intralipid)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>&lt; 40</td>
</tr>
<tr>
<td>+</td>
<td>40 – 99</td>
</tr>
<tr>
<td>++</td>
<td>100 – 199</td>
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</tr>
<tr>
<td>++++</td>
<td>300 – 500</td>
</tr>
<tr>
<td>+++++</td>
<td>&gt; 500</td>
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</table>
Intralipid preparation
Intralipid content

20% soybean oil
1.2% egg yolk phospholipids
2.25% glycerin
water

Major soybean fatty acids
Is Intralipid adequate for interference testing?

- Sample turbidity is only weakly correlated with triglyceride concentration in patient samples.
- The nonlinear relationship between turbidity and triglyceride concentration reflects the heterogeneous nature of lipoprotein particles.
- The L-Index does not accurately reflect triglyceride concentration.
- The structure of synthetic liposomes in Intralipid is very different from chylomicrons.
- Intralipid does not usually mimic interference from triglycerides.
Back to the case

<table>
<thead>
<tr>
<th></th>
<th>T bili</th>
<th>Trig</th>
</tr>
</thead>
<tbody>
<tr>
<td>8/23</td>
<td>24.8</td>
<td></td>
</tr>
<tr>
<td>8/24</td>
<td>9.7</td>
<td>3609 (ultracentrifuged)</td>
</tr>
<tr>
<td>8/25</td>
<td>8.2</td>
<td>2938</td>
</tr>
</tbody>
</table>

| Protein, Total         | 7.1    |                    |
| Albumin                | 5.5    |                    |
| Alkaline Phosphatase   | ↑ 132  |                    |
| Bilirubin, Total       | ↑ 24.8 |                    |
| AST                    | ↑ 51   |                    |
| ALT                    | 52     |                    |
Review of recent cases of hypertriglycerideridemic pancreatitis

<table>
<thead>
<tr>
<th></th>
<th>Tbili</th>
<th>Trig</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.2</td>
<td>&gt; 1000</td>
<td>(ultracentrifuged sample)</td>
</tr>
<tr>
<td>1.2</td>
<td>&gt; 1000</td>
<td></td>
</tr>
<tr>
<td>1.4</td>
<td>5080</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1840</td>
<td></td>
</tr>
</tbody>
</table>
### Hemolysis: in vitro, in vivo?

<table>
<thead>
<tr>
<th>Test</th>
<th>Result</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bilirubin, Total Plasma</td>
<td>2+ HEMOLYSIS</td>
<td>RESULTS REPORTED ON ULTRACENTRIFUGED SAMPLE, EXCEPT TRIGLYCERIDES WHICH ARE PERFORMED ON ORIGINAL SAMPLE</td>
</tr>
<tr>
<td>Bilirubin, Total Plasma</td>
<td>[0.0-1.4 mg/dL]</td>
<td>RESULT IS INVALID BECAUSE HEMOLYSIS INDEX EXCEEDED ALLOWABLE LIMITS.</td>
</tr>
<tr>
<td>LDH</td>
<td>[140-271 U/L]</td>
<td>RESULT IS INVALID BECAUSE HEMOLYSIS INDEX EXCEEDED ALLOWABLE LIMITS. CALLED TO SOPHIA MCCLAIN RN AT 1323 BY GCA ON 05/23/16</td>
</tr>
<tr>
<td>Bilirubin, Total Plasma</td>
<td>[0.0-1.4 mg/dL]</td>
<td>RESULT IS INVALID BECAUSE HEMOLYSIS INDEX EXCEEDED ALLOWABLE LIMITS.</td>
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Hi Dang,

I agree, lipemic interference is heterogeneous for the reasons you mention which is why I would never rule it out without more information. I also agree there is definitely association between hemolysis and lipemia which is interesting.

When you mentioned the bilirubin being elevated due to hemolysis, I initially assumed you meant in vivo hemolysis. As far as I know our current assay is not affected by hemolysis, but then I noticed the lab is not reporting TBili on hemolyzed specimens even though the manufacturer claims there is no interference, so I went over to find out why and it turns out that our old assay was affected by hemolysis. We switched assays in April, 2015, the new assay is the same vendor but slightly reformulated (perhaps partially to remove the hemolysis interference) BUT apparently the vendor made an oversight when they programmed the instrument which the lab discovered by way of clinician complaint back in Jan 2016. They found that hemolyzed specimens were running about 1-3 mg/dL higher than non-hemolyzed specimens from same patients and drawn only a few hours apart.

The vendor came out and reprogrammed the assay in 2016, so theoretically there should no longer be an interference but we are still not reporting TBili on hemolyzed specimens. Now that I am aware we will plan an interference study. That said, in general it is thought that hemolysis should artificially decrease bilirubin due to interference with the formation or stability of the azobilirubin; although this will be assay specific (with our example of the opposite clearly).
Has anyone found that very lipaemic serum samples have high haemolysis indices?

On our high throughput hospital clinical chemistry analysers we measure haemolysis, icterus and lipaemia using simple spectrophotometric indices. This is to avoid analysing tests where these interference are known to lead to factitious results.

We have recently observed that some patients with persistent raised triglycerides have high haemolysis indices and there is a relatively strong correlation!

Has anyone else seen this? I am not sure if this represents increased in vitro haemolysis in lipaemic samples or a direct analytical interference. The strong correlation suggests the later to me?
Jaundice, hyperlipemia and hemolytic anemia: a heretofore unrecognized syndrome associated with alcoholic fatty liver and cirrhosis.

ZIEVE L.

In 1958 Zieve [1] described twenty alcoholic patients with liver disease, hyperlipemia and transient hemolytic anemia and suggested that this combination constituted a new syndrome. Since then other investigators have reported similar cases [2–7] and expressed the same view [8–12].
Maximal bilirubin in chronic hemolysis

Case: Hgb 6, Retic 55%, hapto < 6, LDH 569, T bili 4.2
Summary:

- Artifactual hyperbilirubinemia
- Understanding diazo method in bilirubin determination and potential interference
<table>
<thead>
<tr>
<th>Flag</th>
<th>LIP (mg/dL Intralipid)</th>
<th>ICT (mg/dL Bilirubin)</th>
<th>HEM (mg/dL Hemoglobin)</th>
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<tr>
<td>N</td>
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<td>10 – 19.9</td>
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<td>&gt; 40</td>
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Reagent Constituents

Sodium Benzoate
Caffeine
Sulfanilic acid
HCl
Sodium Nitrite
Sodium Acetate

347 mmol/L
173.9 mmol/L
27 mmol/L
50 mmol/L
0.36 mmol/L
609 mmol/L