Light Has a Greater Effect on Direct Bilirubin Measured by the Bilirubin Oxidase Method than by the Diazo Method

Hiroshi Ishii,1 Yutaka Aoki,1 Tsugutoshi Aoki,1 and Mitsutaka Yoshida2

We compared the effect of light on direct-reacting bilirubin (DBIL) measurement by the bilirubin oxidase (EC 1.3.3.5; BOX) method and by the Jendrassik–Gröf diazo method. DBIL concentrations determined by the BOX method in the sera of hyperbilirubinemic infants treated with phototherapy yielded falsely higher values than those by the direct diazo method. A similar tendency was noted when DBIL concentrations in infants’ sera irradiated with light in vitro were determined by both methods, although by HPLC none of these sera had detectable DBIL (i.e., conjugated plus delta bilirubin). In general, DBIL concentrations after phototherapy remained unchanged when measured by the diazo method, but significantly increased when the BOX method was used. Indeed, phototherapy gave rise to material that acted like a photobilirubin product, which was oxidized at pH 3.7 and therefore was measured as DBIL. Such false increases in DBIL values generated by the BOX method may have clinical diagnostic implications in monitoring jaundiced neonates and in differentiating between physiological jaundice and incipient pathological jaundice.

Additional Keyphrases: phototherapy - jaundice - variation, source of

Recently, total (TBIL) and direct-reacting bilirubin (DBIL) concentrations have been determined enzymatically by using bilirubin oxidase (EC 1.3.3.5, BOX) (1, 2).3 Good agreement has been shown between these TBIL and DBIL values determined by the enzyme-based method and the results of the diazo-based method. However, the BOX-measured DBIL concentration in the sera of several infants treated with phototherapy was several-fold higher than the value given by the direct diazo method. In several cases, the DBIL value by the BOX method increased to pathological values (>10 mg/L) without any indication of hepatobiliary disease.

In this study, we have ascertained more systematically whether the effect of light exposure in vitro was indeed greater on DBIL measurement by the BOX method than by the diazo method.

Materials and Methods

Clinical samples. Serum samples were obtained from 11 icteric newborn infants with no suspected hepatobiliary disease. Six infants were treated with 24 h of phototherapy; the rest were untreated. Phototherapy was administered by means of four 20-W fluorescent lamps mounted on a shelf 60 cm above the bed surface of a standard incubator (Atom phototherapy unit PIT-120; Atom Co., Tokyo, Japan). Blood was sampled immediately after phototherapy stopped.

Photolysis. Sera from hyperbilirubinemic infants not treated with phototherapy were placed separately in a 0.5-cm-pathlength glass cuvette located 3 cm from a 30-W tungsten lamp (Noma-Hitachi, Ltd., Tokyo, Japan) that had a radiation spectrum between 340 and 900 nm. The temperature of the cuvette was maintained at 25°C by cooled air.

Bilirubin measurements. The Jendrassik–Gröf diazo procedure (1, 2), the direct diazo method, was performed with a kit (Bilirubin B II-Test; Wako, Osaka, Japan). "Nescauto TBIL Vg" and "Nescauto DBIL Vg" (Nippon Shoji Kaisha, Ltd., Osaka, Japan) were the reagents in the BOX method (3). We used BOX dissolved in phosphate buffer, pH 7.2, to measure TBIL, and dissolved BOX in citric acid–lactic acid buffer, pH 3.7, to measure DBIL. Bilirubin concentrations were determined from decreases in absorbance at 450 nm.

Assay of photobilirubin. High-performance liquid-chromatographic (HPLC) separation of photobilirubin was performed as described by Kosaka et al. (4) with the use of a Bakerbond wide-pore octyl-C8 column (J.T. Baker, Phillipsburg, NJ). The best separation of photobilirubin was achieved by using a linear gradient of methanol in 100 mL/L dimethyl sulfoxide solution, pH 2.0, containing 10 mmol of tetra-N-butylammonium hydrogen sulfate per liter as counter ion (from 40/60 to 70/30 by vol in 15 min), at 40°C and a flow rate of 1.0 mL/min. The spectrophotometer was set at 450 nm. Serum samples were prepared for analysis by 26-fold dilution with distilled water or BOX-DBIL reagent; 50-μL aliquots of the diluted serum were injected onto the column.

Results

In confirmation of earlier reports (1, 2), both TBIL and DBIL concentrations as determined by the BOX method in the sera of six hyperbilirubinemic infants not treated with phototherapy yielded the same values as determined by the diazo method (Table 1). Exposure to light in vitro led to decreased concentrations of TBIL in sera by both the BOX method and the diazo method; however, TBIL concentrations measured by these methods were in good agreement.

Table 1. Bilirubin Concentrations in Sera from Six Hyperbilirubinemic Infants Not Treated with Phototherapy

<table>
<thead>
<tr>
<th>TBIL, mg/L</th>
<th>DBIL, mg/L</th>
<th>Photobilirubin, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>DiazO</td>
<td>BOX</td>
<td>DiazO</td>
</tr>
<tr>
<td>48</td>
<td>46</td>
<td>4</td>
</tr>
<tr>
<td>52</td>
<td>50</td>
<td>3</td>
</tr>
<tr>
<td>76</td>
<td>74</td>
<td>4</td>
</tr>
<tr>
<td>90</td>
<td>89</td>
<td>3</td>
</tr>
<tr>
<td>156</td>
<td>153</td>
<td>3</td>
</tr>
<tr>
<td>172</td>
<td>172</td>
<td>3</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>99 ± 53</td>
<td>97 ± 53</td>
</tr>
</tbody>
</table>

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3 Nonstandard abbreviations: TBIL, total bilirubin; DBIL, direct-reacting bilirubin; and BOX, bilirubin oxidase.

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HPLC analysis showed the presence of a large peak of unconjugated bilirubin (namely, indirect bilirubin), which was eluted at 17 min, and a small peak with the same retention time as delta bilirubin (13 min), clearly separated in nonirradiated serum. When photoirradiation was applied in vitro, a polar peak with a shorter retention time (6 min) appeared, and the prospective photobilirubin fraction increased in parallel with the irradiation time. After treatment with BOX-DBIL reagent, however, the photobilirubin fraction in irradiated serum disappeared (Figure 2).

TBIL concentrations in five hyperbilirubinemic infants subjected to phototherapy determined by the BOX method were not different from determinations by the diazo method. However, DBIL concentrations of light-treated infants determined by the BOX method were significantly higher than determinations by the diazo method (Table 2). All of the sera revealed the presence of photobilirubin by HPLC, but these fractions disappeared after the treatment with BOX-DBIL reagent. Both photobilirubin fractions present in sera from infants subjected to phototherapy as well as in sera irradiated in vitro were stable for more than 24 h in the dark at 4°C.

Discussion

Exposure of bilirubin to light produces photobilirubin (5) and reduces TBIL in jaundiced neonates (6). Phototherapy is now used as the routine treatment of such patients. So far, the diazo method has been adequate for measuring both TBIL and DBIL concentrations: however, many are now using the BOX method because of its greater specificity for bilirubin (1, 2). Using the BOX method, we have found that DBIL concentrations in photoirradiated infants are high enough to suggest possible physiological jaundice. We hypothesized that this phenomenon was the result of photobilirubin being formed during photoirradiation and that this photobilirubin was being determined as DBIL by the BOX method. The effects of photobilirubin on DBIL concentrations are very small in the diazo method, but relatively large in the BOX method. Because physiological jaundice is characterized by a low DBIL concentration, the false increase in DBIL in the BOX method demonstrated here may present a
Table 2. Bilirubin Concentrations in Sera from Five Hyperbilirubinemic Infants Treated with Phototherapy

<table>
<thead>
<tr>
<th>TBL, mg/L</th>
<th>DBIL, mg/L</th>
<th>Photobilirubin, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diazo BOX</td>
<td>Diazo BOX</td>
<td></td>
</tr>
<tr>
<td>90</td>
<td>87</td>
<td>2</td>
</tr>
<tr>
<td>112</td>
<td>111</td>
<td>2</td>
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<td>120</td>
<td>118</td>
<td>2</td>
</tr>
<tr>
<td>132</td>
<td>130</td>
<td>2</td>
</tr>
</tbody>
</table>

Mean ± SD 114 ± 16 112 ± 16 2 ± 1 13 ± 2* 7 ± 2

* Significantly different: P < 0.001 (Student's t-Test).

Clinical diagnostic problem. Onishi et al. (7) reported that the bilirubin photoisomer in the E-configuration easily reverted to native bilirubin in bile. However, in our study, presumptive photobilirubin is still present in clinical samples and is stable for more than 24 h in serum.

Differential Assay of Zidovudine and Its Glucuronide Metabolite in Serum and Urine with a Radioimmunoassay Kit

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We developed an ancillary procedure for the ZDV-Trac RIA (Incstar) to allow simultaneous determination of both zidovudine (3'-azido-3'-deoxythymidine, ZDV, AZT, Retrovir®) and its metabolite, the glucuronide of ZDV (3'-azido-3'-deoxy-5'-O-β-D-glucopyranuronosylthymidine, ZDVG, GAZT), in human serum and urine. Using the ZDV-Trac RIA, we measured ZDV concentrations before and after ZDV in samples was hydrolyzed to ZDV by β-glucuronidase (EC 3.2.1.31); ZDV glucuronide concentration was calculated as the difference between the two results. This method enables rapid evaluation of a large number of samples with a total turnaround time of 6 h. The lower detection limit of the RIA was 0.27 µg/L; the measurements varied linearly with ZDV concentrations from 0.27 to 217 µg/L, with the 50% inhibitory concentration being ~10 µg/L. Analytical recoveries of in-house serum and urine controls for both ZDV and ZDVG exceeded 90%. Coefficients of variation (CVs) of serum controls were <6% for ZDV and <11% for ZDV; for urine controls, CVs for both ZDV and ZDVG were <6%. Results for ZDVG concentrations obtained by HPLC and by the ZDV-Trac RIA system compared well: r = 0.978, slope 1.0, for serum samples, and r = 0.993, slope 1.09, for urine samples.

Additional Keyphrases: β-glucuronidase • monitoring therapy • azidothymidine (AZT) • acquired immunodeficiency syndrome

Zidovudine (ZDV, AZT, Retrovir®, 3'-azido-3'-deoxythymidine) is an anti-retroviral drug shown to be effective in the treatment of acquired immunodeficiency syndrome (AIDS) and AIDS-related complex (1, 2). ZDV is extensively metabolized to 3'-azido-3'-deoxy-5'-O-β-D-glucopyranuronosylthymidine (ZDVG, GAZT), an inactive metabolite that is rapidly cleared from plasma with an apparent half-life of 1 h (3). The major adverse reaction associated with ZDV administration is bone-marrow suppression (4); thus it is important to assess the efficacy and toxicity of ZDV by monitoring ZDV and ZDVG in biological fluids. Among the techniques described for quantifying ZDV are an HPLC method that measures both ZDV and ZDVG (5), other HPLC systems that measure only ZDV (6, 7), and, as we previously described, a [3H]ZDV-based radioimmunoassay (8), a time-resolved fluorolmmunoassay based on an anti-ZDV polyclonal antiserum, and an enzyme-linked immunosorbent assay based on an anti-ZDV monoclonal antibody (9). Granich et al. (10) recently reported a fluorescence polarization immunoassay for measuring ZDV.

None of the HPLC systems lends itself to high throughput. By contrast, immunoassays can be quick and efficient. A commercial immunoassay kit, ZDV-Trac RIA kit (Incstar Corp., Stillwater, MN), has been introduced for determinations of ZDV in human serum. Here we describe adaptation

References

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2 Incstar Corp., Stillwater, MN 55082.
3 Wellcome Research Laboratories, Beckenham, Kent, U.K. Received December 29, 1989; accepted March 26, 1990.

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Nonstandard abbreviations: ZDV, zidovudine (AZT); ZDVG, 3'-azido-3'-deoxy-5'-O-β-D-glucopyranuronosylthymidine; AIDS, acquired immunodeficiency syndrome; HIV, human immunodeficiency virus; and [3H]ZDV, [3H]-labeled tyramine derivative of ZDV.