"Direct" and Total Bilirubin Tests: Contemporary Problems

John A. Lott\(^1\) and Basil T. Doumas\(^2\)

In eight unique challenges mailed by The College of American Pathologists Comprehensive Chemistry Survey to participating laboratories within 3 years, results for direct-reacting bilirubin (DBIL) were highly variable among the 12 largest peer groups, and most of the results differed greatly from the values obtained by a preferred method. Peer-group mean values for total bilirubin (TBIL) were in much better agreement with each other and with those obtained by the Reference Method for TBIL. From a review of the information on the assay of DBIL provided to us by the manufacturers, we conclude that among the major causes of the large variability and bias in DBIL assays are problems with calibration, lack of a serum blank measurement, inadequate concentrations of HCl in the reaction mixture, inappropriate use of bichromatic correction methods, and possibly the use of wetting agents or surfactants in the reagent. Within-group SDs were small and generally acceptable. The among-peer-group variability in DBIL values is attributable to bias, not imprecision. We recommend several simple changes that could improve the accuracy of DBIL determinations in clinical laboratories.

**Indexing Terms**: ditaurobilirubin \- proficiency testing \- bichromatic spectrophotometry \- intermethod comparison \- accuracy \- variation, source of

In 1989, two lyophilized "wild card" human serum specimens fortified with ditaurobilirubin (DTB) were sent to all of the ~6000 laboratories participating in the College of American Pathologists Comprehensive Chemistry Survey (CAP CCS).\(^3\) The results reported by the participants were surprising indeed: direct bilirubin (DBIL) values between 0.15 and 1.13 mg/dL\(^4\) were reported for a pool fortified to contain DTB at ~0.5 mg/dL (8.6 μmol/L) and values between 0.63 and 2.9 mg/dL for a second pool manufactured to contain ~2.0 mg/dL (34 μmol/L); these results were the highest and lowest means from the 12 largest peer groups in the CCS. The large variations from the expected values were surprising. What went wrong? The list of possible causes of the variability includes the fortifying material (DTB), incidents during manufacture and lyophiliza-

---

\(^1\) Department of Pathology, St. Mary Lying M-368, The Ohio State University, Columbus, OH 43210-1240.

\(^2\) Department of Pathology, Medical College of Wisconsin, Milwaukee, WI 53226.

\(^3\) Nonstandard abbreviations: DTB, ditaurobilirubin; CAP, College of American Pathologists; CCS, Comprehensive Chemistry Survey; DBIL, direct bilirubin; Bu, unconjugated bilirubin; Bc, bilirubin glucuronide; and TBIL, total bilirubin.

\(^4\) The units for DBIL and total bilirubin (TBIL) used by the CAP in the CCS are in the commonly used "mg/dL" units. To convert either DBIL or TBIL in mg/dL to μmol/L, multiply by 17.1.

Received September 22, 1992; accepted November 10, 1992.

---

\(^5\) Addresses of the instrument or reagent manufacturers mentioned here: Abbott Laboratories, North Chicago, IL 60064; Baxter Healthcare Corp., Irvine, CA 92718; Beckman Instruments, Inc., Brea, CA 92621; Boehringer-Mannheim Diagnostics (BMD), Indianapolis, IN 46250; Coulter Electronics, Inc., Hialeah, FL 33012; Du Pont Co., Wilmington, DE 19888; Eastman Kodak Co., Rochester, NY 14650; Instrumentation Laboratory (IL), Lexington, MA 02173; Olympus Corp., Lake Success, NY 11042.
ensure the sum of the two glucuronides (Bc) by a bichromatic method, i.e., by measuring the reflectance at two wavelengths and solving simultaneous equations to estimate the Bc and Bu fractions (2). The Ektachem methods also provide a DBIL value that is the sum of Bc and delta bilirubin.

We present explanations as to why some DBIL results are so inaccurate, and we recommend certain simple measures that should improve the specificity of DBIL assays.

Materials and Methods

DBIL and TBIL Survey Challenges

All the challenges were prepared by Baxter-Dade (Miami, FL 33125) with a bilirubin-free base pool. Unconjugated bilirubin (Sigma Chemical Co., St. Louis, MO 63178) and DTB (Porphyrin Products, Logan, UT 84321) were added according to the "Dade target values" shown in Table 1. Dark amber-colored glass bottles were used for packaging to prevent photodestruction of DBIL. Human-serum-based pools were used in 1989 and 1990. Because these samples were quite cloudy after reconstitution, methods that did not use a specimen blank encountered significant interference. In 1991, human serum albumin was used as the base, to yield specimens that were clearer than the earlier samples after rehydration. An estimate of the absorbance at 598 nm, an indication of the turbidity, is given in Table 1. Human serum albumin has some disadvantages: The reactivities of DTB in human serum albumin, bovine serum albumin, and bovine serum in the direct diazo method are 88%, 76%, and 85%, respectively, of its reactivity in human serum (3).

Proficiency Testing Logistics

Two lyophilized challenges containing DTB and Bu were sent to participants in the CAP CCS in both 1989 and 1990 in the first mailing for that year; they were sent in their own containers and were not part of the main CCS challenges. The instructions were to rehydrate and analyze each specimen for DBIL and TBIL. In 1991, one separate lyophilized challenge in a human serum albumin base was included with each of the four CCS mailings. The participants returned their data to the CAP in Northfield, IL, for processing and statistical analysis. As is the usual CAP custom, extreme outliers (>4 SD from mean) were excluded as possibly representing blunders, transcription errors, and the like. The total number of DBIL and TBIL assays reported per mailing for the "wild card" specimens from 1989 to 1991 are shown in Table 2. The total number of participants in the CCS remained fairly constant, while participation in the DBIL survey grew by about fourfold, probably reflecting increased interest (concern?) in the laboratories' proficiency in performing the DBIL test.

Questionnaire to Manufacturers

We tried to discover the reagent compositions used for DBIL and TBIL assays by the 12 largest peer groups by sending questionnaires to the reagent and (or) instrument manufacturers. The questions are shown in abbreviated form in the left-most column of Table 3. A draft of this Table was later mailed to the manufacturers for verification of the contents and the corrections of errors. Some reagents contain proprietary materials that limit the value of this information.

---

Table 1. CAP Comprehensive Chemistry Surveys. Direct and Total Bilirubin: Manufacturer's Target Values and Analytical Data Obtained by the Preferred Method (4) and the Reference Method (6), Respectively

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Dade target DTB</td>
<td>8.6 (0.50)</td>
<td>34 (2.0)</td>
<td>68 (4.0)</td>
<td>17 (1.0)</td>
<td>26 (1.5)</td>
<td>17 (1.0)</td>
<td>68 (4.0)</td>
</tr>
<tr>
<td>Preferred method a</td>
<td>5.0 (0.29)</td>
<td>16 (0.94)</td>
<td>34 (1.98)</td>
<td>6.2 (0.36)</td>
<td>14 (0.80)</td>
<td>9.9 (0.58)</td>
<td>41 (2.40)</td>
</tr>
<tr>
<td>Preferred method b</td>
<td>21 (1.20)</td>
<td>32 (1.90)</td>
<td>59 (3.45)</td>
<td>23 (1.35)</td>
<td>16 (0.93)</td>
<td>12 (0.68)</td>
<td>43 (2.50)</td>
</tr>
<tr>
<td>Specimen blank c, A</td>
<td>0.058</td>
<td>0.058</td>
<td>0.180</td>
<td>0.135</td>
<td>0.008</td>
<td>0.006</td>
<td>0.006</td>
</tr>
<tr>
<td>Dade target TBIL</td>
<td>17 (1.0)</td>
<td>86 (5.0)</td>
<td>103 (6.0)</td>
<td>34 (2.0)</td>
<td>306 (18)</td>
<td>34 (2.0)</td>
<td>103 (6.0)</td>
</tr>
<tr>
<td>Ref. Method b</td>
<td>17 (1.01)</td>
<td>83 (4.85)</td>
<td>89 (5.22)</td>
<td>24 (1.42)</td>
<td>287 (16.8)</td>
<td>34 (1.96)</td>
<td>100 (5.83)</td>
</tr>
<tr>
<td>Ref. Method c</td>
<td>32 (1.90)</td>
<td>98 (5.75)</td>
<td>111 (6.49)</td>
<td>39 (2.29)</td>
<td>289 (16.9)</td>
<td>35 (2.02)</td>
<td>101 (5.90)</td>
</tr>
</tbody>
</table>

| Specimen blank c, A  | 0.067     | 0.067     | 0.202     | 0.152     | 0.007     | 0.004     | 0.005     | 0.005     |

a Year and challenge no.
b With specimen blank correction.
c Without specimen blank correction.
d Absorbance of the specimen blank at 598 nm vs water in 1-cm cuvettes. Sulfanilic acid is substituted for the diazo reagent. See ref. 4 for details.

---

Table 2. Number of Laboratories Reporting DBIL and TBIL Survey Results to CAP

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>DBIL</td>
<td>1179</td>
<td>1188</td>
<td>2102</td>
<td>2073</td>
<td>3329</td>
<td>3771</td>
<td>4263</td>
</tr>
<tr>
<td>TBIL</td>
<td>3786</td>
<td>3750</td>
<td>3727</td>
<td>3789</td>
<td>3738</td>
<td>4609</td>
<td>5051</td>
</tr>
</tbody>
</table>

---

642 CLINICAL CHEMISTRY, Vol. 39, No. 4, 1993
Table 3. Direct Bilirubin Method Information from Manufacturers

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Preferred method</th>
<th>Abbott Spectrum</th>
<th>Beckman Astra</th>
<th>Beckman CX 4/5</th>
<th>BMD Hitachi 700s</th>
<th>Coulter Decos</th>
<th>Baxter/Dade Paramax</th>
<th>Du Pont cace</th>
<th>Du Pont Dimension</th>
<th>IL Monarch</th>
<th>Kodak 700XR</th>
<th>Olympus Demand</th>
<th>Technicon RA-1000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagents</td>
<td>Doumas</td>
<td>Abbott</td>
<td>Beckman</td>
<td>Beckman</td>
<td>NIST*</td>
<td>NIST 916</td>
<td>Baxter</td>
<td>Du Pont</td>
<td>Du Pont</td>
<td>IL</td>
<td>Kodak</td>
<td>Olympus</td>
<td>Technicon</td>
</tr>
<tr>
<td>Diazo time, s</td>
<td>600</td>
<td>120</td>
<td>25, rate</td>
<td>60-90</td>
<td>240-300</td>
<td>296</td>
<td>60</td>
<td>262</td>
<td>270-310</td>
<td>Spectral</td>
<td>570/600</td>
<td>380/550</td>
<td>66</td>
</tr>
<tr>
<td>Wavelength, nm</td>
<td>598</td>
<td>564/660</td>
<td>545</td>
<td>550-520/630</td>
<td>550/630</td>
<td>540/600</td>
<td>540/700</td>
<td>550/690</td>
<td>600/460/540</td>
<td>60</td>
<td>70</td>
<td></td>
<td>25</td>
</tr>
<tr>
<td>Spectral absorption</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes or no</td>
<td>Yes</td>
</tr>
<tr>
<td>Chloride</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Turbidity interferes</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Hgb, interference, mg/L</td>
<td>500</td>
<td>500</td>
<td>400</td>
<td>1500</td>
<td>250-2000</td>
<td>1000</td>
<td>125</td>
<td>500</td>
<td>2500</td>
<td>250</td>
<td>150</td>
<td>1000</td>
<td>?</td>
</tr>
<tr>
<td>Secret reagents</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes or no</td>
<td>No</td>
</tr>
<tr>
<td>Linear t, mg/dL (µmol/L)</td>
<td>20 (342)</td>
<td>12 (205)</td>
<td>10 (171)</td>
<td>10 (171)</td>
<td>10 (171)</td>
<td>20 (342)</td>
<td>25 (428)</td>
<td>22 (376)</td>
<td>20 (342)</td>
<td>12 (205)</td>
<td>12 (205)</td>
<td>12.6 (215)</td>
<td>12.6 (215)</td>
</tr>
<tr>
<td>OK for neonates</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes or no</td>
<td>Yes</td>
</tr>
<tr>
<td>pH of reaction mixture</td>
<td>-1.5</td>
<td>-1.30</td>
<td>-1.5</td>
<td>-1.5</td>
<td>-1.5</td>
<td>-1.3</td>
<td>3.0</td>
<td>3.3</td>
<td>-1.5</td>
<td>&lt; 2.0</td>
<td>8.15</td>
<td>-1.5</td>
<td>-1.5</td>
</tr>
<tr>
<td>Wetting agent used</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Vol. fraction, final*</td>
<td>0.14</td>
<td>0.038</td>
<td>0.015</td>
<td>0.015</td>
<td>0.045</td>
<td>0.10</td>
<td>0.067</td>
<td>0.012</td>
<td>0.070</td>
<td>0.033</td>
<td>0.021</td>
<td>0.063</td>
<td>?</td>
</tr>
<tr>
<td>HCl, final conc, mmol/L</td>
<td>80</td>
<td>0</td>
<td>54</td>
<td>50</td>
<td>155</td>
<td>330</td>
<td>?</td>
<td>?</td>
<td>74</td>
<td>80</td>
<td>150</td>
<td>92</td>
<td>0.12 g/L Tris*</td>
</tr>
</tbody>
</table>

Unusual reagents: Dichloroaniline; sulfamic acid, 69 mmol/L

Oxalic acid® DPTA® Diphyl line + mordant 0.12 g/L Tris®

Grade: 0 14 2 1 1 7 10 15 1 10 2 14 8

* National Institute of Standards and Technology, Gaithersburg, MD 20899, was the source of unconjugated bilirubin.
* Interference from hemoglobin begins at concentration stated.
* Fraction that is serum in final reaction volume.
* Plus a "tabling agent." Diethylenetriaminepentaacetic acid added as "stabilizer.
* Plus "Armasept," 25 g/L.
* See text. A grade of zero means agreement with the preferred method. The lower the grade, the better the results.
Results and Discussion

The preferred method for DBIL (4) has some limitations; neither conjugated bilirubins nor delta bilirubin reacts quantitatively. The ratios of direct to total bilirubin for DTB, Bc, and delta bilirubin are -0.86 (3), 0.80 (5), and 0.90 (5), respectively. The main advantage of the preferred method is that the contribution of Bu to the observed DBIL is only 1–3% of Bu (4).

Target and Preferred Method Results for DBIL and TBIL

The manufacturer's target values for DBIL and TBIL, and the results for these analytes by the preferred method (4) and the Reference Method (6) assayed with and without a specimen blank, are shown in Table 1. The fact that the values found by analysis are much lower than the "target" values is not surprising. The purity of the purest preparation of DTB—the disodium salt—is 70%. Preparations of Bu (used as the standard in the analysis of DTB) and DTB contain variable amounts of volatile materials (3). Also, DTB does not react quantitatively in the direct diazo method. Thus 1 g of pure DTB is equivalent to no more than 0.6 g of Bu; this proportion could be even less when volatile material and other impurities are considered.

DBIL Results for Largest Peer Groups

In Figure 1, we show the peers' values as ratios of the results to those by the preferred method for all eight challenges. Agreement with the preferred method would give a ratio of 1.0. Intra-group CVs for a given challenge were generally acceptable: ~20% at 20 umol/L, ~15% at 40 umol/L, and ~10% at 70 umol/L. Detailed data on the peers' CVs are available from us. Methods that gave results exceeding those of the preferred method continued to do so for all eight challenges, particularly the Abbott Spectrum, Baxter Paramax, Coulter Dacos, Du Pont Accu, IL Monarch, and the Olympus Demand. The Beckman Astra, Beckman CX 4/5, BMD Hitachi analyzers, Du Pont Dimension, and Kodak Ektachem analyzers obtained DBIL results that were closest to those of the preferred method (with specimen blank) for all of the challenges. The within-pool-group SDs were generally small and acceptable; the problem with the DBIL assay is bias, not imprecision.

The Kodak method for DBIL is unique in that the unreacted Bu and Bc are measured by direct spectrophotometric reflectance at two wavelengths (2). Although results from the Ektachem methods were close to those of the preferred method, it is difficult to say whether the accuracy of the Bu/Bc slide can be tested with specimens containing DTB, because we do not know whether the spectrum of the mordanted Bc is identical to that of DTB. However, on the basis of the low Bu result in specimen C92 for 1991 (which had a high Bu concentration), we conclude that the Ektachem analyzers do not measure Bu as Bc. The Technicon RA-1000 showed a shift in results over the period of this study; it gave DBIL results that were much too high in 1989 and 1990, but their results for 1991 were closer to those of the preferred method. The reason for the improvements is probably the lack of turbidity in the 1991 specimens. The Technicon RA-1000 showed good specificity for DBIL, provided a sample blank was used.

Grading Scheme

We devised a scheme for grading the means of the peers' values, assuming that the DBIL values obtained with the preferred method were correct. For the eight challenges, if the reported result of a given challenge was >2 times or <0.5 times the preferred result (always after blanking), the grade for that challenge is 2. If the reported result was >1.5 times or <0.67 times the preferred result, the grade is 1; if the result was closer than this to the mean determined by the preferred

Fig. 1. Ratio of peers' mean results to the preferred value for DBIL for the eight DBIL CAP challenges mailed in 1989, 1990, and 1991: (top) groups with generally acceptably high values for DBIL (see text); (bottom) groups with generally acceptable DBIL values for the same challenges.

The challenge numbers (abscissa) are in the same chronological sequence as shown in Table 1. (Top) ⊙, Abbott Spectrum; ⊗, Baxter Paramax; ⋄, Coulter Dacos; ⊠, Du Pont Accu; X, IL Monarch; ▲, Olympus Demand, ⊠, Technicon RA-1000, (Bottom) ⊙, Beckman CX 4/5; ⊗, Beckman Astra; ⋄, BMD Hitachi; ⊠, Du Pont Dimension; X, Kodak Ektachem.
method, the grade is 0. A low total grade indicates good agreement with the preferred method. The grade shown in the last row of Table 3 is simply the sum of these. Sixteen is the worst possible grade; zero, the best. Of the 54 nonzero grades, only two received a score because their results were lower than the values obtained by the preferred method. Those with poor grades all overestimated DBIL, some by extraordinarily large amounts.

Total Bilirubin Results for Largest Peer Groups

The peer-group results and the preferred method results (the latter always with a serum blank) for TBIL are in much better agreement than were the corresponding results for DBIL (Figure 2). With minor exceptions, the measurement of TBIL is not the same problem as exists for DBIL, and standardization and analytical problems for TBIL have largely been resolved. We conclude that all 12 peer groups generally produced clinically acceptable TBIL values.

Data on Methods from Manufacturers

Method data for DBIL and TBIL obtained from the manufacturers for the 12 largest peer groups are given in Table 3. There is considerable variability in the DBIL and TBIL methods, and no one uses the preferred method for DBIL or the Reference Method for TBIL (6) in routine work. In our view, the most important factors affecting accuracy in DBIL assays are calibration, concentration of HCl in the final reaction mixture, reaction times, specimen blanking, dichromatic correction techniques, and possibly the presence of wetting agents.

Variables Affecting DBIL Assays

Calibration. DTB is a satisfactory calibrator for DBIL assays with the possible exception of direct spectrophotometric methods. DTB is used in the Ektachem calibrators for the Bc assay; this is done by assigning Bc values to DTB calibrators by an independent method so that patients' DBIL results agree with those of the preferred method (7). The reaction of the diazo reagent with DTB in 50 mmol/L HCl is ~87% complete in the preferred method. However, because DBIL methods in most clinical analyzers are calibrated with DTB, and because DTB is the direct-reacting bilirubin in the CAP specimen, the incomplete reaction should be of no consequence. Given the large discrepancies between DBIL results for some peer groups that use DTB as their standard for the DBIL assay (e.g., Baxter Paramax, IL Monarch), the incomplete coupling of DTB in dilute HCl appears to be a minor problem (3).

Effect of concentration of HCl. At pH <2, Bu does not react in the preferred DBIL assay. If serum is diluted with water instead of with 50 mmol/L HCl, the reactions of DBIL and DTB are more complete but, unfortunately, more Bu reacts as DBIL (4). The reactivity of Bu is further decreased if the specimen is incubated for 5 min in 50 mmol/L HCl before the diazo reagent is added. Peer groups having no or inadequate amounts of HCl in the reaction mixtures reported falsely increased DBIL values. The Baxter Paramax and Du Pont aca use little or no HCl in the reaction mixture, and these peers consistently overestimated the DBIL in the survey challenges. Those peers using DTB as the standard and a pH <2 in the reaction mixture (e.g., Beckman Astra and CX 4/5, Du Pont Dimension, BMD Hitachi) produced results in better agreement with the preferred method.

Reaction times. Reactions times of <1 min in some of the instruments could underestimate DBIL in serum because of the slow-reacting delta bilirubin (5); however, there should be no effect for the CAP survey specimens for peers using DTB as the calibrator for DBIL.

Specimen blank. All serum-based CAP challenges were extremely turbid, the 1990 specimens more so than the 1989 specimens. Thus methods not using a serum blank correction were expected to overestimate both the DBIL and TBIL in the C-94 and C-95 challenges for 1989 and 1990. When the specimen blank correction is omitted, the effect of turbidity is to increase the DBIL or TBIL values by ~0.9 to 1.5 mg/dL (15 to 25 μmol/L).
Turbidity introduces only a small bias in the Bc and TBIL values obtained with the Ektachem analyzers, despite the lack of a specimen blank (8). The insoluble lipids that cause the turbidity are apparently retained on the spreading layer of the film and therefore do not interfere with the reflectance measurement. The Beckman Astra uses a rate method for DBIL to avoid the interference caused by turbidity and nonbilirubin pigments; consequently, their values are closer to those of the preferred method.

The use of human serum albumin in preparing the 1991 specimens' eliminated turbidity. The specimen blank contributed no more than −2 μmol/L (0.1 mg/dL) to the reported value for bilirubin (see Table 1). This observation helped us to identify methods that measured substantial amounts of Bu as DBIL: Abbott Spectrum, Baxter Paramax, Du Pont aca, IL Monarch, and Olympus Demand.

**Bichromatic techniques.** The effect of turbidity in instruments that attempt to correct for background absorbance by measuring the absorbance at two wavelengths—so-called bichromatic measurement—could be either overestimation or underestimation of the bilirubin; the sign of the bias depends on the secondary wavelength used. If the latter is longer than the primary wavelength, the bias will be positive; if shorter, it will be negative. Light-scattering owing to turbidity varies with the wavelength; it decreases as the wavelength increases.

Comparison of bilirubin values obtained with the two versions of the Technicon RA-1000, i.e., single wavelength with correction for sample blank vs bichromatic measurement, demonstrates the effect of turbidity and leaves little doubt that turbidity is also responsible, at least in part, for the high bilirubin values obtained with the Du Pont aca and the Abbott Spectrum. For the Technicon RA-1000, the peers’ mean DBIL for 1989 and 1990 after bichromatic blank correction (and with results for a serum blank shown in parentheses) were 1.13 (0.60), 2.15 (1.63), 4.08 (3.16), and 1.51 (0.72) mg/dL. The values with the bichromatic correction are consistently higher; we conclude that such bichromatic corrections are inappropriate.

**Wetting agents or surfactants.** Surfactants such as Brij-35 cause Bu to react like DBIL (9). Wetting agents in the DBIL reagent were reported by 7 of the 12 peer groups and could account for the falsely increased values obtained by the Coulter Dacos, IL Monarch, Olympus Demand, and the Technicon RA-1000. But wetting agents were also used by the Beckman Astra, Beckman CX 4/5, and the BMD Hitachi groups, which obtained results that were in reasonable agreement with the values by the preferred method.

**Recommendations**

We believe that the most important requirement for a reliable DBIL method is specificity; i.e., the method must not measure Bu as DBIL. This is achieved by diluting the specimen with HCl (~50 mmol/L) and letting it stand for a few minutes before adding the diazo reagent (4). Diluting with acid and preincubating for 5 to 10 min reduces the contribution from Bu to a minimum. With most modern clinical analyzers, incubation of the sample for such times is impractical or unfeasible; however, dilution with HCl to achieve a pH near 1.5 should not be difficult. Surfactants, preservatives, or other additives should generally be avoided. They may be used only if it is ascertained that they do not promote the reaction of Bu in DBIL assays. Measuring absorbance at a single wavelength with proper specimen blanking is preferable and has greater validity than bichromatic measurements. The latter could result in gross errors when specimens are turbid or pigmented. The bichromatic approach is valid only when the background absorbance of the specimen is the same at both wavelengths; it also assumes that the absorbance owing to turbidity, hemoglobin, and any other pigments is the same in the unreacted and diazotized specimen, a situation that never occurs.

Specificity can be tested by analyzing solutions of Bu in pooled human sera by the particular direct method adopted to the analyzer. The Bu concentration in these solutions should range from ~17 to 340 μmol/L (1 to 20 mg/dL). Bovine serum albumin should not be used, because the reactivity of Bu is much greater in this medium than in human serum. Analytical methods that detect >~17 μmol/L (1 mg/dL) DBIL in Bu solutions (such as the above) that are free of DBIL are unreliable and should undergo modifications to correct the problem. As noted in Materials and Methods, the reactivity of DTB in the direct diazo reaction varies with the protein matrix.

The TBIL testing in the peers we surveyed is quite good, with minor exceptions. Standardization and analysis of TBIL in our peer groups were much more accurate than that for DBIL; the latter needs much more attention from instrument and reagent manufacturers and from clinical laboratories to bring the assay to contemporary standards of accuracy and clinical acceptability.

We thank Alfred Hartmann, Chair, Chemistry Resource Committee of the CAP, for his ongoing encouragement and support. We also thank Bernadette Thornton of The Ohio State University Clinical Chemistry Laboratory for her help with the many CAP Survey Challenges and Jendrzejczak of the Medical College of Wisconsin for performing the DBIL and TBIL assays.

**References**

mination of total bilirubin in serum: development and validation. 
The Ektachem clinical chemistry slide for simultaneous determination 

8. Glick MR, Ryder KW, Jackson SA. Graphical comparisons of 
interferences in clinical chemistry instrumentation. Clin Chem 
9. Novros JS, Koch TR, Knoblock EC. Improved method for 
accurate quantitation of total and conjugated bilirubin in serum. 