Direct Spectrophotometric Method for Measurement of Bilirubin in Newborns: Comparison with HPLC and an Automated Diazo Method, *Steven C. Kazmierczak,[1*] Alex F. Robertson,[2] Paul G. Catrou,[3] Kimberly P. Briley,[3] Bill L. Kreamer,[4] and Glenn R. Gourley[4]* ([1] Department of Pathology, Oregon Health and Sciences University, 3181 SW Sam Jackson Park Rd., Portland, OR 97201; Departments of [2] Pediatrics and [3] Pathology and Laboratory Medicine, Brody School of Medicine of East Carolina University, Greenville, NC 27858; [4] Department of Pediatrics and Waisman Center, University of Wisconsin, 1500 Highland Ave., Madison, WI 53705; * address correspondence to this author at: Department of Pathology, Oregon Health and Sciences University, Mailcode L471, 3181 SW Sam Jackson Park Rd., Portland, OR 97201; fax 503-494-8148, e-mail kazmierc@ohsu.edu)

The measurement of serum total bilirubin is one of the most frequently performed tests in newborns (1–3). Direct spectrophotometry for the measurement of bilirubin in sera from newborns is a simple and rapid method that requires minimal sample for analysis (4). The direct spectrophotometric assay is based on the absorbance of bilirubin at 454 nm; by contrast, hemoglobin absorbs equally at both 454 and 528 nm. Subtraction of the absorbance at 528 nm from that at 454 nm eliminates the effect of hemolysis and yields a value that can be attributed primarily to bilirubin. Unfortunately, other pigments, such as carotenoids, also absorb at 454 nm, thus limiting this technique to neonates <2–3 weeks of age.

Direct spectrophotometric methods have been compared with diazo (Jendrassik–Gróf-based) bilirubin methods (5–9), but sample hemolysis can increase or decrease the results obtained by the latter procedure, depending on the concentrations of hemoglobin and bilirubin in the sample (10, 11). Most such studies either do not describe the effect of sample hemolysis on the agreement between methods or report poorer agreement between methods or increased scatter around the regression line (6, 8, 9).

HPLC for measurement of serum bilirubin concentrations is labor-intensive and not practical for routine use; however, it is not subject to interference from hemoglobin or lipemia. We used HPLC to investigate a direct spectrophotometric procedure for the measurement of bilirubin in the plasma of neonates and measured the hemoglobin concentrations in all samples to assess the impact of hemolysis.

This study was performed after approval by the Institutional Review Boards of both participating hospitals. Informed consent was obtained from mothers before collection of blood from newborns. Thirty-seven blood samples were obtained from neonates <4 days of age (mean age, 48 h; range, 12–86 h). The samples were collected into heparin-containing, amber-colored containers after heel puncture with a capillary puncture device (Tenderfoot®; International Technidyne Corp.). No specimens were collected from infants undergoing phototherapy. The samples were immediately sent to the hospital laboratory, where the plasma was separated and then divided into two aliquots. One aliquot was immediately assayed for bilirubin for clinical purposes by a diazo method in an AU640 analyzer (Olympus). The second aliquot was placed in an amber-colored container and frozen at −70 °C for subsequent analysis of hemoglobin and bilirubin by HPLC and bilirubin by a direct spectrophotometric method.

We measured bilirubin concentrations by HPLC according to a previously described method (12). Hemoglobin and bilirubin concentrations were measured simultaneously by HPLC, except that the diode-array wavelength of detection was 404 nm (4 nm bandwidth) with a reference of 464 nm (4 nm bandwidth). Hemoglobin solutions (Sigma-Aldrich) were used to construct an appropriate calibration curve. While the HPLC analysis was being performed, aliquots of these same samples were used to measure bilirubin concentrations by a direct spectrophotometric method with a bilirubinometer (LEICA UNISTAT®; Leica Inc.). The Leica instrument measures the bilirubin concentration in 20 μL of serum without dilution. Absorbance is measured at 460 and 550 nm and is used to calculate the total bilirubin concentration in the sample. A weekly calibration was performed with the use of an assayed glass calibration cuvette. Total imprecision (CV) according to the manufacturer is ~15% and 10% at bilirubin concentrations of 21 μmol/L (1.2 mg/dL) and 130 μmol/L (7.6 mg/dL), respectively, and the reportable range is 0.0–684.0 μmol/L (0.0–40.0 mg/dL). The time required for bilirubin analysis with this instrument is ~15 s.

The median bilirubin concentration measured in the 37 specimens by HPLC was 197 μmol/L (11.5 mg/dL) [range, 46.2–295.8 μmol/L (2.7–17.3 mg/dL)]. A regression analysis performed according to the Deming method (13) between the HPLC (x) and Olympus analyzer (y) results yielded a slope (SD) of 0.93 (0.07) and a y-intercept (SD) of 13.7 (13.7) μmol/L (r = 0.89). The Deming regression analysis for the HPLC (x) vs Leica bilirubinometer (y) results yielded a slope (SD) of 1.06 (0.03) and a y-intercept (SD) of −9.4 (5.3) μmol/L (r = 0.99). The scatter around the regression line was much less for the Leica bilirubinometer vs the HPLC results ($S_{y|x}$ = 5.4 μmol/L) compared with the Olympus analyzer vs HPLC results ($S_{y|x}$ = 16.3 μmol/L). The mean squares of the residuals were significantly different (F = 8.9; P <0.001).

Bland–Altman (14) difference plots (Fig. 1) revealed little dependence of the disagreement between methods on bilirubin concentration. However, the plots readily showed greater disagreement between bilirubin concentrations measured by HPLC and the Olympus analyzer compared with the HPLC vs Leica bilirubinometer methods. The maximum differences between the HPLC and the Olympus analyzer results varied from −42.8 μmol/L (−2.5 mg/dL) to 59.9 μmol/L (3.5 mg/dL), with a mean (SD) difference of −1.2 μmol/L (22.3 μmol/L). The maximum differences between the HPLC and the Leica bilirubinometer results varied from −24.0 μmol/L (−1.4 mg/dL) to 15.4 μmol/L (0.9 mg/dL), with a mean (SD) difference of −1.7 μmol/L (8.3 μmol/L).

Virtually all samples in this study showed evidence of hemolysis. The median hemoglobin concentration was 35.7 μmol/L [230 mg/dL; range, 8.7–197.6 μmol/L (56–1275 mg/dL)]. Because of the complex nature of the mechanism of hemoglobin interference in bilirubin measurements, there was no direct relationship between hemoglobin concentrations and the magnitude and direction of the interference bias. A plot of the hemoglobin concentration in each sample vs the magnitude of the interference bias, calculated as HPLC-measured bilirubin minus Olympus analyzer- or Leica analyzer-measured bilirubin concentrations, revealed a slope and y-intercept of 0.001 and −7.5 μmol/L, respectively, for the Olympus analyzer procedure and 0.000 and −2.1 μmol/L, respectively, for the Leica bilirubinometer procedure. Neither slope was statistically significantly different from zero ($P > 0.99$).

In conclusion, the measurement of total bilirubin with a Leica direct spectrophotometric procedure provides bet-

ter agreement with HPLC than does a diazo (Jendrassik–Gróf-based) procedure in an Olympus analyzer, and the Leica method provides the advantages of small instrument size, small sample volume, and rapid turnaround. However, it should be noted that none of the specimens in this study was visibly lipemic, and we did not evaluate the effects of lipemia on the Leica and Olympus methods. In addition, all samples were obtained from newborns <4 days of age.

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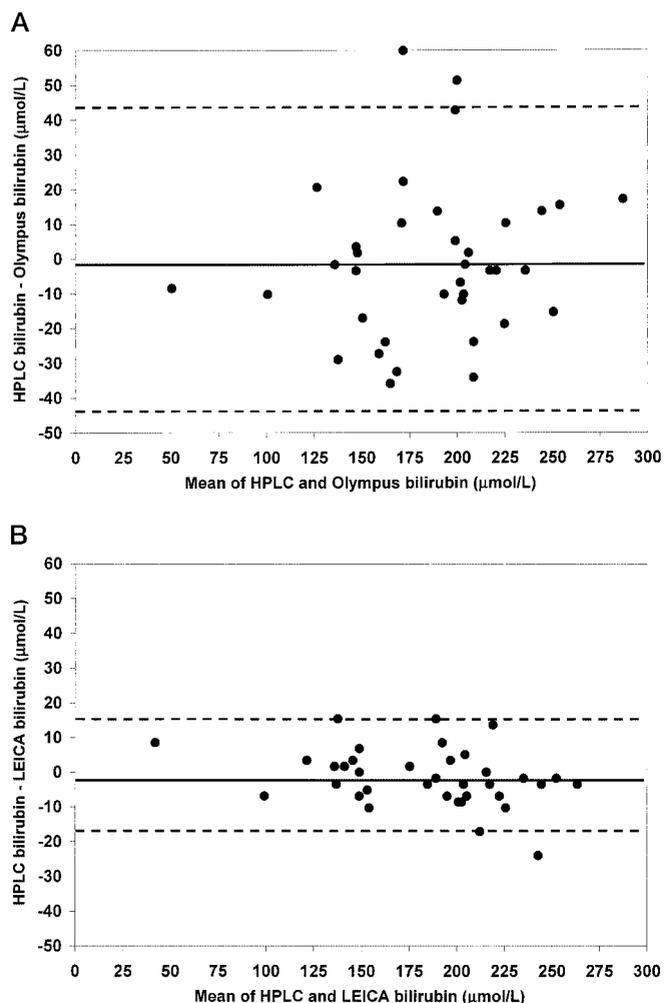**A**

B

Fig. 1. Bland–Altman plots of bilirubin results obtained by HPLC vs Olympus analyzer (*A*) and HPLC vs Leica bilirubinometer (*B*).

The *solid line* represents the mean difference in measured bilirubin concentrations between methods, and the *dashed lines* are ± 2 SD.

Release Characteristics of Cardiac Biomarkers and Ischemia-modified Albumin as Measured by the Albumin Cobalt-binding Test after a Marathon Race, *Fred S. Apple,* *Heidi E. Quist, Angela P. Otto, Wendy E. Mathews, and MaryAnn M. Murakami* (Department of Laboratory Medicine and Pathology, Hennepin County Medical Center, University of Minnesota School of Medicine, Minneapolis, MN 55415; * address correspondence to this author at: Clinical Laboratories MC 812, Hennepin County Medical Center, 701 Park Ave., Minneapolis, MN 55415; fax 612-904-4229, e-mail fred.apple@co.hennepin.mn.us)

Numerous studies have monitored the appearance of both cardiac and skeletal muscle proteins and enzymes after short- and long-term exercise regimens. Both animal and human exercise models have attempted to determine whether the stress of long-duration exercise, such as a