〈Brief Note〉

# Realization of the total and direct reaction of ditaurobilirubin via the use of a modified Jendrassik-Grof diazo procedure

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**Summary** Ditaurobilirubin (DTB), a synthetic water-soluble bilirubin derivative, has been suggested as a possible surrogate calibration standard for the assay of conjugated bilirubin in serum. Herein, to assign DTB solution concentration, we quantified total and direct-reacting bilirubin (TBIL and DBIL, respectively) using the Jendrassik-Grof diazo procedure. In the prepared DTB solution (100.0 mg/L), TBIL concentration was determined as 95.4% of the predicted value, while DBIL concentration was estimated as 65.0% (62.0 mg/L) of TBIL concentration. When the employed procedure was modified by replacing hydrochloric acid with acetate buffer as an acidic medium, DBIL concentration increased to 93.5% (89.2 mg/L). TBIL concentration was concluded to reflect the concentration of the DTB solution, while DBIL concentration was concluded to be reagent composition-dependent. Consequently, discrepancies between TBIL and DBIL values possibly observed for DTB-based calibrators should be considered for recently developed bilirubin measurement methods (i.e., those employing bilirubin oxidase or vanadate).

**Key words:** Total bilirubin, Direct-reacting bilirubin, Diazo reaction, Reference measurement procedure, Calibrator

## 1. Introduction

Currently, the diazo method is globally accepted as a reference measurement procedure (RMP) for total bilirubin (TBIL) quantitation.[1] However, the lack of an RMP and reference materials for the quantitation of direct-reacting bilirubin (DBIL) has necessitated the use of unconjugated bilirubin (UCB) as a calibrator for DBIL assaying. In such measurements, the calibrator (UCB) must be analyzed by a procedure employed for TBIL in the presence of an accelerator that renders UCB water-soluble and diazo-positive.

Recently, ditaurobilirubin (DTB), a synthetic water-soluble bilirubin derivative that directly reacts

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without an accelerator, has been used as a control or calibration material for DBIL quantitation.[2] Herein, the assayed content of the calibration material prepared from DTB was certified as TBIL content via the use of the RMP. This approach poses the general question of whether the DBIL concentration determined in the DTB calibrator is identical to the corresponding TBIL value. Although most bilirubin measurement methods employed in Japan are oxidation method with bilirubin oxidase[3,4] or vanadate,[5] their calibration material prepared from DTB needs to be certified via the diazo method. In this study, the TBIL and DBIL concentrations of a DTB solution were determined using the Jendrassik-Grof diazo procedure and compared.[6]

## 2. Materials and methods

### 2.1. Materials

*Bilirubin solutions:* UCB powder was purchased from Sigma-Aldrich Co., St. Louis, MO, USA (B4126: 98%, *M*w = 584.7 g/mol), and DTB powder was purchased from Frontier Scientific, Inc., Newark, DE, USA (B850: 97%, *M*w = 842.91 g/mol). Bovine serum albumin (BSA), crystallized was sourced from Wako Pure Chemical Industries, Ltd., Osaka, Japan (016-15096: 95%, *M*w = 66000 g/mol).

To prepare the UCB solution (100.0 mg/L after correction for 98% purity), UCB powder (10.2 mg) was dissolved in 0.5 mL of dimethyl sulfoxide and 1.0 mL of 0.1 mol/L aqueous $Na_2CO_3$, and the obtained solution was diluted with 40 g/L BSA dissolved in 0.1 mol/L Tris buffer, pH 7.4 up to 100 mL. The above procedure was identical to that employed in the RMP.[1]

To prepare the DTB solution, DTB powder (14.9 mg) was dissolved in 0.1 mol/L Tris buffer (pH 8.5, 100 mL) containing 40 g/L BSA. The purity of thus prepared solution was determined as 97% by high-performance liquid chromatography,[7] and DTB concentration was calculated as 100.0 mg/L after correction for 97% purity and conversion to UCB equivalent value (via multiplication by 584.7/842.9). DBIL concentration was expressed in terms of UCB equivalent concentration.

### 2.2. Reagents

All assay reagents except for hydrochloric acid, acetate buffer, and ascorbic acid solution were prepared according to the RMP method.[1] Hydrochloric acid and ascorbic acid solution were prepared as required by the Jendrassik-Grof diazo procedure.[6]

*Caffeine-benzoate reagent:* Anhydrous sodium acetate (56 g), sodium benzoate (56 g), disodium EDTA (1.0 g), and caffeine (37.5 g) were dissolved in distilled water (1000 mL).

*Alkaline tartrate solution:* NaOH (75 g) and potassium sodium tartrate (320 g) were dissolved in distilled water (1000 mL).

*Sulfanilic acid solution:* A solution of sulfanilic acid (5.0 g) in distilled water (700 mL) was treated with conc. hydrochloric acid (15 mL) and diluted to 1000 mL with distilled water.

*$NaNO_2$ solution:* $NaNO_2$ (5 g) was dissolved in distilled water (1000 mL).

*Diazo reagent (diazotized sulfanilic acid solution):* $NaNO_2$ solution (1.0 mL) was mixed with sulfanilic acid solution (40 mL) immediately before use.

*Hydrochloric acid:* 0.05 mol/L.

*Acetate buffer:* 0.4 mol/L, pH 4.75.

*Ascorbic acid solution:* Ascorbic acid (200 mg) was dissolved in distilled water (5 mL).

### 2.3. Methods

TBIL (Table 1) and DBIL (Table 2) were quantified according to the Jendrassik-Grof diazo procedure.[6] The absorbance of the alkaline azopigment was measured spectrophotometrically at 598 nm using a Hitachi U-7012 clinical spectrophotometer. TBIL and DBIL concentrations in the DTB solution were determined using the UCB solution (100.0 mg/L) as a calibration material (i.e., standard solution).

## 3. Results and discussion

When TBIL and DBIL concentrations in the

Table 1 TBIL quantitation procedure.

| Reagent | Volume (mL) |
|---|---|
| Caffeine-benzoate solution | 2.0 |
| UCB or DTB solution | 0.25 |
| Diazotized sulfanilic acid solution | 0.5 |
| Mix and wait 10 min at 25 °C | |
| Ascorbic acid solution | 0.1 |
| Alkaline tartrate solution | 1.5 |
| Hydrochloric acid | 1.0 |
| Read the absorbance at 598 nm against blank solution | |

The given reagents need to be added in the above order. For blank absorbance measurement, diazotized sulfanilic acid solution should be substituted by sulfanilic acid solution.

Table 2 DBIL quantitation procedure.

| Reagent | Volume (mL) |
|---|---|
| Hydrochloric acid | 1.0 |
| DTB solution | 0.25 |
| Diazotized sulfanilic acid solution | 0.5 |
| Mix and wait 10 min at 25 °C | |
| Ascorbic acid solution | 0.1 |
| Alkaline tartrate solution | 1.5 |
| Caffeine-benzoate solution | 2 .0 |
| Read the absorbance at 598 nm against blank solution | |

The given reagents need to be added in the above order. For blank absorbance measurement, diazotized sulfanilic acid solution should be substituted by sulfanilic acid solution.

Table 3 TBIL and DBIL concentrations in the DTB solution determined by the Jendrassik-Grof diazo procedure.

| TBIL (mg/L) | DBIL (mg/L) | |
|---|---|---|
| | Hydrochloric acid as an acidic medium | Acetate buffer as an acidic medium |
| 95.4 ± 7.2 | 62.0 ± 2.7 | 89.2 ± 3.1 |

All values are reported as mean ± standard deviation of five measurements.

DTB solution were measured by the Jendrassik-Grof diazo procedure using the 100.0 mg/L UCB solution as a standard, discrepancies between TBIL and DBIL values were observed (Table 3). Although the TBIL concentration was determined as 95.4 mg/L (i.e., 95.4% of the predicted concentration), a much lower value was obtained for the DBIL concentration (65.0% of the TBIL concentration). Although the Jendrassik-Grof diazo procedure employed 0.05 mol/L hydrochloric acid as an acidic medium, Doumas et al.[2] suggested that the usage of acetate buffer (0.4 mol/L, pH 4.75) instead of hydrochloric acid may increase the reactivity of DTB toward the diazo reagent. Herein, this substitution resulted in an increase of the DBIL concentration (Table 3) to values close (93.5%) to the TBIL concentration ($P$= 0.20), which implied that the Jendrassik-Grof diazo procedure underestimated the DBIL concentration. Therefore, control materials prepared from DTB represented two different values of TBIL and DBIL (TBIL ≠ DBIL). We concluded that the TBIL value corresponds to the true DTB concentration, while the DBIL value is variable and depends on the reactivity of the users' employing diazo reagent.

This finding has important implications for the Jendrassik-Grof diazo procedure and other recently developed bilirubin quantitation methods, i.e., those based on oxidation with bilirubin oxidase[3,4] and vanadate[5]. Consequently, to apply DTB solution as a "manufacturer's product calibrator"[8] for TBIL measurement, one needs to certify the calibrator-assigned value employing the diazo-based TBIL method. When the DBIL value of the calibrator is confirmed to be identical to the TBIL value (DBIL = TBIL), both of which are determined by the "manufacturer's standing measurement procedure"[8], the calibrator can be used as a "manufacturer's product calibrator" for DBIL measurement.

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*Disclosure of conflicts of interest*

The authors have no conflicts of interest.

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