〈Brief Note〉

# Determination of molar absorption coefficient of surrogate calibration materials prepared for total and direct bilirubin measurements

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**Summary** Measurements of total bilirubin (TB) and direct-reacting bilirubin (DB) use unconjugated bilirubin (UCB) and ditaurobilirubin (DTB) as calibration standards. The calibration materials UCB (National Institute of Standards and Technology SRM 916a) for TB and DTB lot 41 (Porphyrin Products) for DB are no longer available. Therefore, the molar absorption coefficients of azo pigments in Sigma-Aldrich Co. UCB and Frontier Scientific, Inc. DTB were determined to establish their suitability as surrogate materials. Solutions of Sigma-Aldrich UCB and Frontier Scientific DTB were prepared according to the dissolving method described in the literature to the solute concentration of 171 μmol/L. The molar absorption coefficients of their azo pigments were determined by the diazo method and compared to the corresponding literature value (7664 $m^2$/mol). The molar absorption coefficients of Sigma-Aldrich UCB and Frontier Scientific DTB were 7313 ± 70 and 6970 ± 83 $m^2$/mol (95.4 and 90.9% of the literature value), respectively. By correcting the solute concentration of 171 μmol/L for the values determined for the surrogates (7313/7664 and 6970/7664 for Sigma-Aldrich UCB and Frontier Scientific DTB, respectively), these surrogates may potentially be used in lieu of the abovementioned traditional calibration materials.

**Key words:** NIST SRM 916a, Bilirubin, Ditaurobilirubin, Molar absorption coefficient, Reference measurement procedure

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## 1. Introduction

Serum bilirubin is classified into four fractions when analysed via high-performance liquid chromatography: unconjugated bilirubin (UCB), bilirubin monoglucuronide, bilirubin diglucuronide, and delta bilirubin[1]. However, in clinical usage, it is typically measured only as total bilirubin (TB) and direct-reacting bilirubin (DB)—TB being the sum concentration of all four fractions and DB that of the latter three fractions based on their reactivity with diazo reagent. TB measurements are typically calibrated with a crystalline UCB powder from the National Institute of Standards and Technology (NIST), Gaithersburg, MD, USA (Standard Reference Material SRM 916a)[2], and DB measurements are typically conducted using ditaurobilirubin, disodium salt (DTB lot 41)[3] from Porphyrin Products (Logan, UT, USA). The concentrations of these calibration materials have been certified based on the molar absorption coefficients of their alkaline azo pigments at a wavelength of 598 nm[2-4].

Despite its use as a standard reference material and the lack of an available alternative, NIST SRM 916a has not been produced or sold by the NIST for several years. Similarly, DTB lot 41 is no longer available from Porphyrin Products. Therefore, we investigated alternate preparations of UCB and DTB as surrogates for those from NIST and Porphyrin Products, respectively. The aim of this study was to determine how the molar absorption coefficients of these surrogates compare to those of NIST SRM 916a and Porphyrin Products DTB lot 41.

## 2. Materials and Methods

### Reagents

As respective surrogates for NIST SRM 916a and Porphyrin Products DTB lot 41, UCB powder was purchased from Sigma-Aldrich Co., St. Louis, MO, USA (B4126: 98% purity, $M_w$ = 584.66 g/mol) and DTB powder (lot JH16-11040) from Frontier Scientific, Inc., Newark, DE, USA (B850: $M_w$ = 842.91 g/mol). Sigma-Aldrich UCB powder (porcine gall origin) and Frontier Scientific DTB powder (chemically synthesized) are available worldwide. In 1999, the company "Porphyrin Products" changed its name to "Frontier Scientific". Although we could not identify whether DTB lot 41 was taken over by DTB lot JH16-11040, the difference in purity between lot 41 (88%)[3] and lot JH16-11040 (97%, described later) implies that lot JH16-11040 may be an upgraded material. UCB and DTB solutions were prepared using the same dissolving methods as those used to produce NIST SRM 916a[2,4] and Porphyrin Products DTB lot 41[3], respectively, to final solute concentration of 171 μmol/L after correcting for purity.

For the UCB solution, 10.2 mg of Sigma-Aldrich UCB powder was weighed out on a plastic weighing dish (not on glassine paper) using Sartorius Entris 64-1S analytical balance (Sartorius Weighing Technology GmbH, Gottingen, Germany). It was transferred to a 100-mL amber volumetric flask, and was dissolved in 0.5 mL dimethyl sulfoxide and 1.0 mL 0.1 mol/L aqueous sodium carbonate, followed by dilution with 40 g/L crystallized bovine serum albumin (BSA, 016-15096: 95% purity, $M_w$ = 66000 g/mol, Wako Pure Chemical Industries, Ltd., Osaka, Japan) dissolved in 0.1 mol/L Tris buffer (pH 7.4) to a volume of 100 mL. Since bilirubin is light-sensitive, UCB and DTB solutions were prepared in subdued light (away from windows and with the laboratory light off) at 20–25°C, as per usual precautions. Powders stuck to the dish should be washed into the flask by using dimethyl sulfoxide (UCB) or Tris buffer (DTB).

For the DTB solution, 14.9 mg of the Frontier Scientific DTB powder was dissolved in 100 mL 0.1 mol/L Tris buffer (pH 8.5) containing 40 g/L BSA. The purity of the DTB solution was determined to be 97% via high-performance liquid chromatography analysis[5]. The percentage purity was calculated by comparing the area of the main peak and total peak areas of the impurities.

### Measurement procedure

The measurement procedure, including all assay reagents, followed the candidate reference

method (hereafter the reference measurement procedure, RMP) for TB measurement proposed by Doumas et al.[4] (Table 1). The absorbance of alkaline azo pigments in the Sigma-Aldrich UCB and Frontier Scientific DTB solutions was spectrophotometrically measured with a Hitachi U-5100 UV/Vis Ratio Beam Spectrophotometer (wavelength 598 ± 1 nm, bandwidth 5 ± 0.5 nm, path length 10 mm), and the molar absorption coefficients at 598 nm ($\varepsilon$, $m^2$/mol) were calculated with Eq. (1). This is the same procedure as that used to certify the molar absorption coefficients of the azo pigments in NIST SRM 916a and Porphyrin Products DTB lot 41[2,3].

$$\varepsilon = A_{598} \times [V_t/V_s] \times C_b^{-1} \times d^{-1} \quad (1)$$

where $A_{598}$ is the absorbance at 598 nm, $V_t$ is the total volume (L), $V_s$ is the sample volume (L), $C_b$ is the bilirubin concentration (mol/L), and $d$ is the path length (m).

The molar absorption coefficients of azo pigments in Sigma-Aldrich UCB and Frontier Scientific DTB were determined across the same lot of three vials. Each determination was achieved on a different day using an unopened vial.

Statistics

The data were analyzed using Student's *t*-test, and differences of $p < 0.05$ were considered to be statistically significant.

## 3. Results and Discussion

The molar absorption coefficient of the azo pigment in NIST SRM 916a was certified in 1990 by five laboratories[6] as 7649 ± 21 $m^2$/mol and reassessed in 2006 by the NIST itself[2] as 7664 ± 63 $m^2$/mol. However, because of the unavailability of NIST SRM 916a, the coefficient has not been evaluated since (to our knowledge). Therefore, although Klauke et al.[7] used 7649 $m^2$/mol, we used 7664 $m^2$/mol as reference value as this is the value listed in the NIST SRM 916a data sheet. We determined that the molar absorption coefficient of the azo pigment in Sigma-Aldrich UCB was 7313 ± 70 $m^2$/mol (95.4% of the literature value, $p < 0.05$) (Table 2).

Table 1 Measurement procedure based on the candidate reference method.

| Reagent | Volume (mL) |
|---|---|
| Caffeine-benzoate solution | 2.0 |
| UCB or DTB solution | 0.25 |
| Diazotized sulfanilic acid solution[1] | 0.5 |
| Mix and wait 10 min at 25°C | |
| Alkaline tartrate solution | 1.5 |
| Read the absorbance at 598 nm against blank solution | |

The given reagents must be added in the above order.

[1]For blank absorbance measurement, the diazotized sulfanilic acid solution should be substituted by sulfanilic acid solution.

Table 2 Molar absorption coefficients of azo pigments formed from Sigma-Aldrich UCB and Frontier Scientific DTB.

| | Sigma-Aldrich UCB ($m^2$/mol) | Frontier Scientific DTB ($m^2$/mol) |
|---|---|---|
| Vial 1 | 7237 ± 111 | 6878 ± 121 |
| Vial 2 | 7374 ± 113 | 7041 ± 106 |
| Vial 3 | 7328 ± 110 | 6990 ± 77 |
| Average | 7313 ± 70 | 6970 ± 83 |

Values are mean ± standard deviation of five measurements across the same lot of three different vials for UCB and DTB.

Hence, laboratories can use Sigma-Aldrich UCB as a possible surrogate for NIST SRM 916a by correcting the observed value by 7664 $m^2$/mol. We certified the concentration of the UCB solution ($C_{UCB}$, μmol/L) using Eq. (2).

$$C_{UCB} = 171 \times (7313/7664) \quad (2)$$

Alternatively, UCB concentration in another solution different from 171 μmol/L was certified using Eq. (3).

$$C_{UCB} = [V_t/V_s] \times (A_{598} / 7664) \times 10^5 \quad (3)$$

where $[V_t/V_s]$ is the dilution factor, and $A_{598}$ is the absorbance at 598 nm. When $[V_t/V_s]$ = *4.25/0.25* = 17 (Table 1) and $A_{598}$ = 0.386, $C_{UCB}$ is calculated to be 86 μmol/L.

Doumas et al.[3] proposed that the molar absorption coefficient of azo pigment in Porphyrin Products DTB lot 41 is identical to that of UCB. The actual value of ε was not presented in the report. Therefore, we compared the ε value of Frontier Scientific DTB to that of NIST SRM 916a (7664 $m^2$/mol). The molar absorption coefficient of Frontier Scientific DTB was determined to be 6970 ± 83 $m^2$/mol (90.9% of the literature value, $p < 0.05$) (Table 2). Hence, in DB measurements, Frontier Scientific DTB can be used as a calibration material after correcting the solute concentration (171 μmol/L) by 6970/7664. Alternatively, $C_{DTB} = [V_t/V_s] \times (A_{598}/7664) \times 10^5$.

We examined another certification for Sigma-Aldrich UCB and Frontier Scientific DTB with solute concentration of 171 μmol/L. They were diluted 10-fold with BSA (40 g/L dissolved in 0.1 mol/L Tris buffer, with pH 7.4 for UCB and pH 8.5 for DTB), and their absorbances were measured at 460 nm via direct spectrophotometry without the addition of a diazo reagent. Consequently, the molar absorption coefficient at 460 nm was determined to be 6096 ± 40 $m^2$/mol for Sigma-Aldrich UCB (without correcting for 7313/7664) and 4773 ± 31 $m^2$/mol for Frontier Scientific DTB (without correcting for 6970/7664). The observed value for DTB was 84.2% of the literature value (5670 $m^2$/mol, $p < 0.05$) [3], but the reference value for UCB dissolved in dimethyl sulfoxide and/or BSA was not reported. The molar absorption coefficient of UCB at 460 nm has been reported as 4910 ± 69 $m^2$/mol dissolved with sodium carbonate and diluted with 0.9% saline in a human serum matrix[8] or as 4700 ± 130 $m^2$/mol dissolved with sodium hydroxide and diluted with 0.1 mol/L phosphate buffer, with pH 7.4 in a human serum albumin matrix[9]. The large discrepancy between the literature values and our observed values requires further investigation, and certification via direct spectrophotometry could not be used at this time. Since molar absorption coefficient of UCB was reportedly elevated to 6000 ± 58 $m^2$/mol upon dissolving in chloroform[8], dimethyl sulfoxide as an organic solvent should have the same effect on UCB. However, this has not yet been verified.

The bilirubin measurement methods recently developed in Japan (i.e., the oxidase method with bilirubin oxidase[10,11] or vanadate[12]) use UCB and/or DTB as calibration materials for TB and DB measurements, respectively. These methods are widely used in Japan, whereas the diazo method is rarely used. Herein, the magnitude of the intra-method coefficient of variation for TB was reported to converge (< 10%), but that for DB varied largely (> 10%) in the Accuracy Management Survey of Clinical Examinations[13] conducted by the Kyushu Quality Control Study Group on Laboratory Medicine. Large intra-method variation was observed when DB reagents that did not react with delta bilirubin (one component of DB) were excluded from the statistics. The results of this study suggest that the RMP combined with NIST SRM 916a could help manufacturers to provide TB reagents to standardize their TB values. Moreover, because no RMP for DB has been developed, one manufacturer used DTB as a calibration material for DB measurement, but another did not use DTB. The latter manufacturer adjusted their DB values to the corresponding values observed by the Jendrassik–Gróf DB assay[14]. These different calibration methods

could have resulted in the large intra-method variation in the DB measurement mentioned above.

A limitation of this study is that the observed $\varepsilon$ values for UCB and DTB had higher standard deviations than those in a previous report[2]. Because direct spectrophotometry presented lower standard deviations against the same preparations, these higher standard deviations could have been caused by technical imprecision. Reagents used in this RMP foamed more easily, and its procedure comprised a three-step manual method that may have caused analytical variation. Moreover, both UCB and DTB are photosensitive. Therefore, at least five measurements under dim light may help to attain more accurate results.

In conclusion, Sigma-Aldrich UCB and Frontier Scientific DTB could potentially be used as surrogates for NIST SRM 916a and Porphyrin Products DTB lot 41, respectively, after correcting their solute concentrations by the molar absorption coefficient of azo pigment (7664 $m^2$/mol) at 598 nm.

## Conflicts of interest

The authors have no conflicts of interest.

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## References

1. Lauff JJ, Kasper ME and Ambrose RT: Separation of bilirubin species in serum and bile by high-performance reversed-phase liquid chromatography. J Chromatogr B Biomed Sci Appl, 226: 391–402, 1981.
2. Gonzalez CA and Choquette SJ: National Institute of Standards and Technology. Certificate of Analysis, Standard Reference Material 916a, Bilirubin, 2016.
3. Doumas BT, Wu TW, Poon KC and Jendrzejczak B: Chemical nature of a synthetic bilirubin conjugate and its reactivities in the total and direct reactions by the Jendrassik-Gróf method. Clin Chem, 31: 1677–1682, 1985.
4. Doumas BT, Kwok-Cheung PP, Perry BW, Jendrzejczak B, McComb RB, Schaffer R and Hause LL: Candidate reference method for determination of total bilirubin in serum: development and validation. Clin Chem, 31: 1779–1789, 1985.
5. Osawa S, Sugo S, Yoshida T, Yamaoka T and Nomura F: An assay for separating and quantifying four bilirubin fractions in untreated human serum using isocratic high-performance liquid chromatography. Clin Chim Acta, 366: 146–155, 2006.
6. Doumas BT, Perry BW, McComb RB, Kessner A, Vader HL, Vink KL, Koedam JC and Paule RC: Molar absorptivities of bilirubin (NIST SRM 916a) and its neutral and alkaline azopigments. Clin Chem, 36: 1698–1701, 1990.
7. Klauke R, Kytzia HJ, Weber F, Grote-Koska D, Brand K and Schumann G: Reference measurement procedure for total bilirubin in serum re-evaluated and measurement uncertainty determined. Clin Chim Acta, 481: 115–120, 2018.
8. Meites S and Traubert JW: Use of bilirubin standards. Clin Chem, 11: 691–699, 1965.
9. Lee KS and Gartner LM: Spectrophotometric characteristics of bilirubin. Pediatr Res, 10: 782–788, 1976.
10. Otsuji S, Mizuno K, Ito S, Kawahara S and Kai M: A new enzymatic approach for estimating total and direct bilirubin. Clin Biochem, 21: 33–38, 1988.
11. Kurosaka K, Senba S, Tsubota H and Kondo H: A new enzymatic assay for selectively measuring conjugated bilirubin concentration in serum with use of bilirubin oxidase. Clin Chim Acta, 269: 125–136, 1988.
12. Tokuda K and Tanimoto K: A new method of measuring bilirubin in serum by vanadic acid [Jpn]. Jpn J Clin Chem (*Rinsho Kagaku*), 22: 116–122, 1993.
13. Takeshita T, Ikeda K, Hamasaki N and Okabe H: An analysis of serum direct bilirubin (D-BIL) data obtained in a control survey conducted by the Kyushu Rinshou Seidokanri Kenkyukai for quality assessment of clinical laboratory [Jpn]. JJCLA, 31: 40–47, 2006.
14. Jendrassik J and Grof P: Vereinfachte photometrische Methoden zur Bestimmung des Bilirubins [Ger]. Biochem Z, 297: 81–89, 1938.