Preparation of bilirubin standard solutions for assay calibration

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Method Article

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Abstract

Bilirubin (BR) is the product of cellular heme catabolism and the major bile pigment in animal blood. It is an established biomarker of hemolysis and liver function. In the last decade, mild hyperbilirubinemia has been shown to be a biomarker of lower cardiovascular disease risk, attributed to its antioxidant and anti-inflammatory effects. To use bilirubin as a predictive biomarker, new powerful methods are currently being developed for its direct analysis in human blood. To harmonize the different methods, it is essential to use high-quality BR standard solutions for assay calibration. We present here a protocol for the preparation of stable standard solutions in the range of 10^{-9} - 10^{-5} M BR at pH 7.4 that can facilitate a universal approach to assay calibration.

Introduction

The pigment bilirubin is an endogenous compound formed in animals by heme catabolism, catalyzed by heme oxygenase and biliverdin reductase¹. Bilirubin is a linear tetrapyrrole in which two carboxyl groups form six intramolecular hydrogen bonds with nitrogen atoms that fix the molecule in its typical ridge-tile 3D configuration. Hydrogen bond-breaking solvents, especially dimethyl sulfoxide², dissolve bilirubin most effectively. In aqueous solution at a physiological pH of 7.4, the solubility of bilirubin decreases to 10^{-9} M. In plasma, bilirubin ranges from 3 - 15 μM ¹ and is almost completely bound to serum albumin (K_d = 17 nM)³.

Analysis of bilirubin in clinical laboratories is performed by automated methods⁴. For highly sensitive analysis of bilirubin in complex biological matrices, a number of other analytical methods have been introduced, based on HPLC and coupled with advanced detectors, various sensors, molecularly imprinted surfaces, or the fluorescent bilirubin-binding protein UnaG⁵⁻⁷.

To harmonize the different methods used in experimental medicine and biology, it is important to use high-quality BR standard solutions for assay calibration, which could facilitate a universal approach to bilirubin analysis.

We present here a protocol for the preparation of stable standard solutions in the range of 10^{-9} - 10^{-5} M bilirubin determined by UV-vis spectroscopy (10^{-5} M) or by the HUG-based fluorometric assay (10^{-9} M)⁸.

TECHNICAL NOVELTIES

1. The standard solutions are in saline solution buffered at physiological pH (not in alkali, not in apolar solvents).

2. The standard solutions are in a wide range of concentrations.
3. The preparation has a quality control procedure based on direct analysis of bilirubin UV-VIS spectra or fluorescence emission of the HUG -bilirubin complex (no colorimetric reaction).

Reagents

Ultrapure-water milliQ for the preparation of all solutions

Dimethyl sulfoxide (DMSO) (Sigma Aldrich, SHBM2271)

Sodium chloride (NaCl) (Sigma-Aldrich)

Potassium chloride (KCl) (Sigma-Aldrich)

Hydrochloric acid (HCl) (Sigma-Aldrich)

Sodium hydroxide (NaOH) (Sigma-Aldrich)

Potassium dihydrogen phosphate (KH_2PO_4) (Sigma-Aldrich)

Disodium hydrogen phosphate dihydrate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$) (Sigma-Aldrich)

Bilirubin (BR) (Sigma-Aldrich, B4126)

Bovine Serum Albumin, Fraction V (BSA) (SIGMA, A-7906)

HELP-UnaG (HUG) fusion protein, in-house production⁸

SOLVENTS

Acronyms and composition:

- DMSO: pure
- PBS: Phosphate Buffered Saline, (136.9 mM NaCl, 2.7 mM KCl, 10.0 mM, $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 1.8 mM KH_2PO_4 , milliQ water), pH 7.4
- PBS-BSA: PBS containing $4 \text{ g} \cdot \text{L}^{-1}$ BSA (60.6 μM BSA), pH 7.4
- PBS-BSA_{dil}: PBS containing $0.4 \text{ g} \cdot \text{L}^{-1}$ BSA (6.06 μM BSA), pH 7.4

Equipment

LABWARE

- Beaker (0.5 L)
- Duran Laboratory glass bottles (500 mL and 250 mL)
- Amber glass vials (10 mL)
- Eppendorf tubes (1.5 mL) (Sarstedt)
- Screw cap tube (15 or 50 ml) (Sarstedt)
- Tips (10 mL, 1 mL, and 200 µL) (Sartstedt)
- 96-well black polystyrene plates (Nunc®)
- Gilson Pipetman Classic Variable Volume Pipette, 2-20 µL
- Gilson Pipetman Classic Variable Volume Pipette, 10-200 µL
- Gilson Pipetman Classic Variable Volume Pipette, 100-1000 µL
- Gilson Pipetman Classic Variable Volume Pipette, 1-10 mL
- Aluminium foil
- Lab Tube Racks
- Stainless steel spoon
- Laboratory tweezers stainless steel
- PTFE Magnetic Stirrer Bar
- Quartz cuvette l = 1cm

INSTRUMENTS

- Milli Q, Millipore Co., Bradford, MAMicrobalance (Mettler Toledo, XS205 Dual range)
- Magnetic stirrer (Icamag® Rec-G)
- pHmeter (Meterlab PHM240)

- Spectrophotometer (Cary 4E, Varian)
- Microplate reader (Synergy, BioTeck Winooski, VT)
- Mettler Toledo XS205 Balance.

Procedure

PREPARATION OF BILIRUBIN STANDARD SOLUTIONS

Bilirubin (BR) solutions can be prepared in amber glass vials or in aluminum-coated screw cap tubes (Table 1).

Solution A

Weigh a few mg of the dry BR powder in an Eppendorf tube on a microbalance.

Add an appropriate volume of DMSO to obtain a 5 mM solution and vortex.

Store 20 μ L aliquots of this solution in Eppendorf tubes at -20 °C.

Stability: 4 months at -20 °C.

Solution B1

Dilute 10 μ L *Solution A* in 4990 μ L PBS to obtain a 10 μ M solution and vortex.

Dilute 500 μ L *Solution B1* in 4500 μ L PBS to obtain a 1 μ M solution and vortex.

Stability: <10 min. Use and analyze it immediately after preparation.

Solution B2

Dilute 10 μ L *Solution A* in 4990 μ L PSB-BSA 4 g·L⁻¹.

Dilute 500 μ L *Solution B2* in 4500 μ L PBS to obtain a 1 μ M solution and vortex.

Stability: 2 h at T = 25°C; wait at least 30 min before using this solution.

Standard Solutions C1

Dilute serial volumes of *Solution B1* (1 and 10 μM) in PBS to a final volume of 5 mL.

The volumes are given in Table 1.

Stability: Use and analyse immediately after preparation (within 5 minutes).

Standard Solutions C2

Dilute serial volumes of *Solution B2* (1 and 10 μM) in PBS-BSA $0.4 \text{ g}\cdot\text{L}^{-1}$ to a final volume of 5 mL.

The volumes are given in Table 2.

Stability: Use and analyse the solution within 1 hour of preparation.

QUALITY CONTROL OF BILIRUBIN SOLUTIONS

1) UV-VIS Spectroscopy

- o Add 3 mL *Solution B1* or *Solution B2* to a quartz cuvette ($l = 1 \text{ cm}$).
- o Prepare a set of $n = 4$ samples.
- o Use PBS or PBS-BSA as blank.
- o Record UV-VIS spectra in the range $350 < \lambda < 600 \text{ nm}$ and find $A_{\text{max}} = 440 \text{ nm}$ and 465 nm for *Solution B1* and *B2*, respectively.
- o BR solutions should be considered accurate if the absorbance value is:

§ *Solution B1*: $0.446 (\pm 0.05)$ at $\lambda_{\text{max}} = 440 \text{ nm}$

§ *Solution B2*: $0.636 (\pm 0.05)$ at $\lambda_{\text{max}} = 465 \text{ nm}$

2) HUG-based Fluorometric assay ⁸

- o Transfer 200 μL of *Standard Solution C1* or *C2* to a black 96-well plate containing 10 μL of 1 g/L (or 16.6 μM) HUG (final volume = 210 μL ; final concentration HUG = 0.79 μM).

- o Fill 5 wells for each BR concentration.
- o Use 200 μL of the solvent of either C1 or C2 solutions (PBS or $\text{PBS-BSA}_{\text{dil}}$).
- o Incubate the covered plate at room temperature ($T = 25\text{ }^{\circ}\text{C}$).
- o Reaction time:
 - 1 hour for *Standard Solution C1*
 - 2 hours for *Standard Solution C2*.
- o Instrumental settings: $\lambda_{\text{ex}} = 485\text{ nm}$, $\lambda_{\text{em}} = 528\text{ nm}$, $T = 25\text{ }^{\circ}\text{C}$, gain 100, reading height 2.50 mm.

3) Range of applicability

- o $7 < \text{pH} < 10$
- o The same calibration curve is obtained at $T = 25^{\circ}\text{C}$ or 37°C
- o BSA concentration does not affect the stability and solubility of BR in standard solution or the kinetics of binding with HUG in a wide range of concentrations ($0.4 - 40\text{ g}\cdot\text{L}^{-1}$).

4) Data Analysis

- o Calculate the mean $\pm$ standard deviation for each BR concentration ($n = 5$).
- o Fit the data of standard curves by linear regression analysis.

Troubleshooting

The dissolution process of BR in physiological buffer is a critical requirement for biochemical assays. Many precautions must be taken when preparing aqueous BR solutions to avoid oxidative reactions and aggregation.

- o Avoid direct light (work in dim light, use amber glass bottles; wrap all tubes and vessels in aluminum foil).
- o Limit exposure to air, by plugging tubes immediately after adding solutions.

- o Avoid excessive shaking, which leads to bilirubin aggregation.
- o Thaw *Solution A* only once; do not reuse it.
- o Perform all experiments with freshly prepared solutions, taking into account the time period during which the solutions are stable.
- o BR solutions prepared in PBS (*Solution B1*) must be analyzed spectrophotometrically within 10 minutes
- o BR solutions prepared in PBS-BSA (*Solution B2*) must be thoroughly mixed and analyzed after at least 30 minutes, the minimum time required to achieve signal stability.
- o The actual concentration of 10 μM BR solutions can be checked by the diazo method ⁹.
- o BR solutions (1 – 50 nM) in PBS (*Solutions C1*) are unstable and must be immediately mixed with HUG.
- o The reaction temperature is defined as room temperature $T = 25 \pm 2^\circ\text{C}$.
- o Cover 96-well plates during the reaction time, to prevent solvent evaporation and photo-degradation of bilirubin.
- o Soak the glassware overnight in aqueous NaOH solution (0.1 M); rinse with aqueous HCl solution (0.1 M) followed by milliQ water.
- o Do not re-use plastic tubes as BR may adsorb to the walls.
- o Sources of error or deviation are:
 - § Uncalibrated pipettes;
 - § Inaccuracy in transferring concentrated solutions to other solvents when preparing dilutions, as some bilirubin may remain on the outer surface of the pipette tips;
 - § Preparation of BR solutions at temperatures and times not specified above;
 - § Exposure of standard solutions to light during preparation.

Time Taken

- From preparation of standard solution in PBS to starting incubation: 10 min
- From preparation of standard solution in PBS-BSA to starting incubation: 40 min

- Incubation time for standard solution in PBS: 1 h at 25°C
- Incubation time for standard solution in PBS-BSA: 2 h 25°C
- The overall time needed to complete the HUG-based fluorometric analysis: approximately 3 hours.

Anticipated Results

Identical standard solutions can be obtained by a single stock solution stored at -20° C for 4 months.

Standard solutions can be prepared in saline solutions buffered at physiological pH, spanning 10^{-5} to 10^{-9} M without or with BSA.

BR standard solutions without BSA have lower absorbance (*Solution B1*) and fluorescence (*Solution C1*) than identical solutions with BSA (*Solutions B2 and C2*).

BR standard solutions with BSA (*Solutions B2 and C2*) are stable at 25°C for 2 hours.

Assessment of BR standard solutions with BSA (*Solutions B2*) by UV-VIS spectroscopy correlates with the assessment by the diazo reaction ⁹.

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Figures

Table 1 List of bilirubin standard solutions.

Solution	[BR]	Solvent	Analytics	Notes on use
A	5 mM	DMSO	UV-VIS	Stock <u>solution</u>
B1	1 and 10 μ M	PBS	UV-VIS	Pre-calibrator solution
B2	1 and 10 μ M	PBS-BSA	UV-VIS	Pre-calibrator solution
C1	1 - 50 <u>nM</u>	PBS	Fluorescence	Fluorescence Calibrator
C2	1 - 50 <u>nM</u>	PBS-BSA _{dil}	Fluorescence	Fluorescence Calibrator

Figure 1

Table 1

Table 2. Scheme for the preparation of bilirubin standard solutions in the concentration range 1-50 nM without (series C1) or with BSA (series C2).

		Standard solutions C1			Standard solutions C2		
STD #	[Br] (nM)	1 μM BR B1 (μL)	10 μM BR B1 (μL)	PBS (μL)	1 μM BR B2 (μL)	10 μM BR B2 (μL)	PBS-BSA (μL)
1	1	5		4995	5		4995
2	2	10		4990	10		4990
3	4	20		4980	20		4980
4	6	30		4970	30		4970
5	8	40		4960	40		4960
6	10	50		4950	50		4950
7	15		7.5	4992.5		7.5	4992.5
8	20		10	4990		10	4990
9	25		12.5	4987.5		12.5	4987.5
10	30		15	4985		15	4985
11	40		20	4980		20	4980
12	50		25	4975		25	4975

Figure 2

Table 2