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Preparation and validation of nanomolar aqueous bilirubin standard solutions ☆,☆☆,★

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ABSTRACT

Bilirubin (BR) is the product of cellular heme catabolism and the main bile pigment in animal blood. It is an established biomarker for hemolysis and liver function. Over the last decade, mild hyperbilirubinemia has been shown to be a biomarker for a lower risk of cardiovascular disease, due to its antioxidant and anti-inflammatory effects. In order to use bilirubin as a predictive biomarker, new powerful methods for its direct analysis in human blood are currently being developed. 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 uniform approach for assay calibration without or with a sample preparation step.

- The bilirubin standard solutions are prepared in buffered saline solution at physiological pH (not in alkali, not in apolar solvents) added with BSA
- The standard solutions are in a wide range of concentrations.
- The preparation has a quality control procedure based on direct analysis of bilirubin UV–VIS spectra or fluorescence emission of the its complex with the recombinant fusion protein HELP-UnaG (HUG).

Specifications table

Subject area:	Biochemistry, Genetics and Molecular Biology
More specific subject area:	*Clinical Biochemistry*
Name of your method:	Preparation of nanomolar aqueous bilirubin standard solutions
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Resource availability:	Reagents and Equipment are listed in the Materials and Reagents section

☆ **Related research article:** None
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Background

The pigment bilirubin is an endogenous compound formed in animals by heme catabolism catalyzed by heme oxygenase and biliverdin reductase [1]. 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 sulfoxide [2], dissolve bilirubin most effectively . In aqueous solution at a physiological pH of 7.4, the solubility of bilirubin drops to 10^{-9} M. In plasma, bilirubin is between 3 and 15 μM [1] and is almost completely bound to serum albumin (K_d = 17 nM) [3].

The analysis of bilirubin in clinical laboratories is performed by automated methods [4]. For the highly sensitive analysis of bilirubin in complex biological matrices obtained in experimental biology and medicine, a number of other analytical methods, based on HPLC coupled with advanced detectors, various sensors, molecularly imprinted surfaces, or the fluorescent bilirubin-binding protein UnaG have been introduced [5–8].

To harmonize the different methods used in clinical biochemistry, experimental medicine and biology, it is important to use high-quality BR standard solutions for assay calibration, which could facilitate a uniform approach to bilirubin analysis as well as method comparison, when assessing real samples that may or may not require a pre-analytical preparation step [9,10].

We present here a protocol for the preparation of stable bilirubin 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) [11,12].

Method details

Materials and reagents

All reagents were purchased from Merck, unless otherwise specified.

- Dimethyl sulfoxide (DMSO)
- Methanol (MeOH)
- Sodium chloride (NaCl)
- Potassium chloride (KCl)
- Hydrochloric acid (HCl)
- Sodium hydroxide (NaOH)
- Potassium dihydrogen phosphate (KH_2PO_4)
- Disodium hydrogen phosphate dihydrate ($Na_2HPO_4{\cdot}2H_20$)
- Bilirubin (BR)
- Bovine Serum Albumin, Fraction V (BSA)
- HELP-UnaG (HUG) fusion protein, in-house production [13]

Equipment

Labware

- Amber glass vials (10 mL)
- Eppendorf tubes (1.5 mL)
- Screw cap tube (15 or 50 ml)
- Tips (10 mL, 1 mL, and 200 μL)
- Black 96-well plates (Nunc®, purchased by Thermofisher, code 237,107; polystyrene, sterile, non–treated surface)
- Variable Volume Pipette, 2–20 μL
- Variable Volume Pipette, 10–200 μL
- Variable Volume Pipette, 100–1000 μL
- Variable Volume Pipette, 1–10 mL
- Aluminium foil
- Lab Tube Racks
- PTFE Magnetic Stirrer Bar
- Quartz cuvette l = 1 cm

Instruments

- Multi-purpose water purification system (Crystal EX, Androna$^{\text{Ò}}$)
- Microbalance (Mettler Toledo, XS205 Dual range)
- Magnetic stirrer (Icamag® Rec-G)
- pHmeter (Meterlab PHM240)
- Double-beam spectrophotometer (CARY–4E UV–visible spectrophotometer, Cary Instruments, Monrovia, Calif. 91,016)
- Microplate reader (Synergy H1; BioTek, Winooski, VT, USA)

Recipes

- PBS: Phosphate Buffered Saline, (136.9 mM NaCl, 2.7 mM KCl, 10.0 mM, $Na_2HPO_4 \cdot 2H_2O$, 1.8 mM
- KH_2PO_4, milliQ water), pH 7.4
- PBS-BSA: PBS containing 4 g/L BSA (60.6 μM BSA), pH 7.4
- PBS-BSA_{dil}: PBS containing 0.4 g/L BSA (6.06 μM BSA), pH 7.4
  The pH of PBS solutions might be adjusted by addition of 1 N HCl or 1 N NaOH.

Software

GraphPad Prism 10.1.0 (264) (GraphPad Softwares, Boston, MA, USA).

Procedure

The dissolution process of BR in physiological buffers is a critical requirement. Many precautions must be taken in the preparation of aqueous BR solutions to avoid oxidative reactions and aggregation.

Preparation of bilirubin standard in aqueous solutions containing bovine serum albumin

These standards can be used in assays where bilirubin (BR) can be completely dissociated from serum albumin under mild (solvent-free) conditions, as is typical for assays based on UnaG fluorescence. Therefore, the expected measurement(s) concern the sum of albumin-bound bilirubin and albumin free-bilirubin. The same standard solutions are suitable for the measurement of both bilirubin glucuronide and biliverdin, provided they are converted to bilirubin by prior incubation with β-glucuronidase [14] or biliverdin reductase [15], respectively.

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

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 μL aliquotes of this solution in Eppendorf tubes at −20 °C.
Stability: 4 months at −20 °C.

Avoid direct light (work in dim light, use amber glass bottles; wrap all tubes and vessels in aluminum foil).
Limit exposure to air by capping tubes immediately after adding solutions.
Avoid excessive shaking as this will lead to bilirubin aggregation.
Thaw Solution A only once; do not reuse it.

Solution B10

Dilute 10 μL Solution A in 4990 μL PSB-BSA.
Stability: 2 h at T = 25 °C; wait at least 30 min before using these solutions.

Solution B1

Dilute 500 μL Solution B10 in 4500 μL PBS-BSA.
Stability: 2 h at T = 25 °C.

Solutions C

Dilute serial volumes of Solutions B1 and B10 in PBS-BSA_{dil} to a final volume of 5 mL.
The volumes are given in Table 2.
Stability: Use and analyse the solutions at 25 °C within 1 h of preparation.

The volume of the solutions can be scaled to a more appropriate amount depending on the experimental requirements.
Perform all experiments with freshly prepared solutions, taking into account the period of time during which the solutions are stable.
Soak the glassware overnight in aqueous NaOH solution (0.1 M); rinse with aqueous HCl solution (0.1 M) and then with milliQ water.
Do not reuse plastic tubes, as BR can adsorb on the walls.

Table 1
List of bilirubin standard solutions.

Solution	[BR]	Solvent	Analytics	Notes on use
A	5 mM	DMSO	UV–vis	Stock solution
B10	10 μM	PBS-BSA	UV–VIS	Pre-standard solution
B1	1 μM	PBS-BSA	UV–VIS	Pre-standard solution
C	1–50 nM	PBS-BSA_{dil}	Fluorescence	Standard Solutions

Table 2
Scheme for the preparation of bilirubin standard solutions in the concentration range 1–50 nM with BSA.

Solutions C in PBS-BSA 0.4 g/L				
STD #	[BR] (nM)	1 μM BR (μL)	10 μM BR (μL)	PBS-BSA (μL)
1	1	5		4995
2	2	10		4990
3	4	20		4980
4	6	30		4970
5	8	40		4960
6	10	50		4950
7	15		7.5	4992.5
8	20		10	4990
9	25		12.5	4987.5
10	30		15	4985
11	40		20	4980
12	50		25	4975

Preparation of deproteinized bilirubin standard solutions

These standards can be used in assays where bilirubin (BR) must be completely dissociated from serum albumin under harsh conditions, resulting in deproteinization of the sample, as is typical for HPLC assays.

Solution B10

Prepare 10 μM solution of BR in PBS-BSA pH 7.4 as described above.
Stability: 2 h at T = 25 ˚C; wait at least 30 min before using this solution.

Solutions C_{MeOH}

Dilute serial volumes of Solutions B (10 μM) in PBS-BSA to a final volume of 0.5 mL. The volumes are given in Table 3.
Stability: Use and analyse the solutions at 25 ˚C within 1 h of preparation.
Take 100 μL of each solution (Table 3), add 200 μL of pure methanol and vortex.
Centrifuge at 10,000 rpm (8600 g) for 5 min.
Add 50 μL of each supernatant to 950 μL of HUG 0.05 g/L to obtain the standards solutions in the range 1 – 50 nM.

The sources of error or deviation in the preparation of all above standard solutions 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.

Quality control of bilirubin solutions

(1) **Solution B10**:

- Add 3 mL Solution B10 to a quartz cuvette (l = 1 cm).

Table 3
Scheme for the preparation of bilirubin solutions in the concentration range 60 –720 nM.

#	BR (nM)	10 μM BR (μL)	PBS-BSA (μL)
1	60	3	497
2	120	6	494
3	180	12	488
4	240	18	482
5	300	24	476
6	360	30	470
7	420	45	455
8	480	60	440
9	540	75	425
10	600	90	410
11	660	120	380
12	720	150	350

- Prepare a set of $n = 4$ samples.
- Use PBS or PBS-BSA as blank.
- Record UV–VIS spectra in a double-beam spectrophotometer (CARY–4E UV–visible spectrophotometer, Cary Instruments, Monrovia, Calif. 91,016) at $T = 25$ °C using quartz cuvettes (Suprasil 10 0.01 mm, Helma Cells Inc., Jamaico, NY 11,424, USA) in the range $350 < \lambda < 600$.
- BR solutions should be considered accurate if the absorbance value is [12]:
- Solution B10: 0.636 (± 0.05) at $\lambda_{max} = 465$ nm

BR solutions prepared in PBS-BSA (Solution B10) must be thoroughly mixed and analyzed after at least 30 min*, the minimum time required to achieve signal stability.*

(2) **Solutions C**

- Transfer 200 µL of Standard Solution C 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).
- Fill 5 wells for each BR concentration.
- Use 200 µL of the solvent of either C solutions (PBS-BSA_{dil}) as blank.
- Incubate the covered plate for 2 h at room temperature (T = 25 °C):
- Read the fluorescence in a benchtop multiplate reader (Synergy H1; BioTek, Winooski, VT, USA) at $\lambda_{ex} = 485$ nm, $\lambda_{em} = 528$ nm (T = 25 ± 2 °C; gain 100, reading height 2.50 mm) [12].

(3) **Solutions C_{MeOH}**

- Transfer 200 µL of Standard Solution C_{MeOH} to a black 96-well plate
- Fill 5 wells for each BR concentration.
- Use 200 µL of the solvent (methanol 5 % in HUG 0.05 g/L) as blank.
- Incubate the covered plate for 2 h at room temperature (T = 25 °C)
- Read the fluorescence in a benchtop multiplate reader (Synergy H1; BioTek, Winooski, VT, USA) at $\lambda_{ex} = 485$ nm, $\lambda_{em} = 528$ nm (T = 25 ± 2 °C; gain 100, reading height 2.50 mm).

Cover 96-well plates during the reaction time, to prevent solvent evaporation and photo-degradation of bilirubin.

Method validation

The bilirubin standards in physiological buffer solutions require the presence of a solubilizing and stabilizing agent [16–18]. To substantiate this, we present below a stability test as proposed in Sist et al. 2023 [12] with minor modifications, comparing bilirubin standards in PBS without and with BSA. For this purpose, we diluted the 5 mM stock solution in DMSO, directly in 50 mL PBS pH 7.4 and for comparison in PBS-BSA_{dil} pH 7.4, as described above, to obtain a final concentration of 50 nM. This final concentration was chosen to be within the BR solubility range in aqueous solution (from 7 nM to 100 nM at pH 7.4, [2,19,20]).

Seven standard solutions (1, 5, 10, 20, 30, and 40 nM) were prepared in PBS or PBS-BSA_{dil} from the 50 nM solution and stored in the dark at 4 °C for the duration of the experiment. At various time points between 0 and 96 h, the solutions were added to a multiwell plate prefilled with 10 µL HUG 1 mg/mL in each well. The fluorescence produced was recorded after 2 h of incubation at $T = 25$ °C. The values of the angular coefficients of the calibration curves obtained at different time points are shown in Fig. 1.

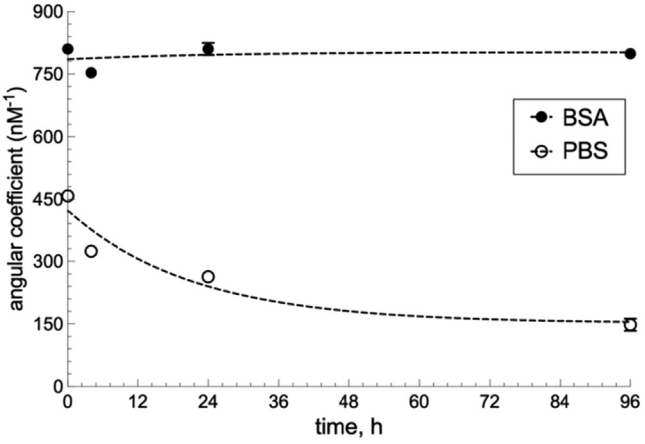

Fig. 1. Stability of BR standard solutions in PBS without or with 0.4 g/L BSA.

Table 4
Recovery of bilirubin in the standards without and with proteins precipitation.

BR Level, nM	Expected **BSA** nM	Observed **MeOH** nM	Recovery %	*p*-value
1	1.05 (± 0.05)	1.06 (± 0.09)	101	>0.999
2	1.58 (± 0.26)	1.69 (± 0.09)	107	>0.999
4	3.60 (± 0.21)	3.50 (± 0.27)	97	>0.999
6	4.98 (± 0.08)	5.28 (± 0.80)	106	>0.999
8	7.66 (± 0.82)	7.33 (± 0.47)	96	>0.999
10	9.20 (± 1.03)	8.87 (± 0.31)	96	>0.999
15	13.67 (± 1.30)	13.69 (± 0.61)	100	>0.999
20	19.54 (± 0.95)	19.05 (± 0.26)	97	>0.999
25	25.06 (± 0.82)	25.11 (± 0.28)	100	>0.999
30	28.47 (± 2.01)	29.94 (± 1.95)	105	0.661
40	37.56 (± 0.60)	38.39 (± 0.88)	102	0.991
50	46.70 (± 0.99)	46.65 (± 4.03)	100	>0.999

It can be seen that the presence of albumin both solubilizes and stabilizes bilirubin in solution, whereas in PBS only 60 % of bilirubin was dissolved and the latter fraction underwent an exponential decay. Therefore, BR solutions (1–50 nM) in PBS are unstable.

The need for stable BR standard solutions exists in all analytical methods, including spectrometry and chromatography. In these methods, it is often necessary to precipitate proteins with methanol prior to BR analysis. The HUG method was used to check the recovery of BR in the nanomolar range after such pre-treatment of the sample. In detail, serial bilirubin solutions were prepared in the concentration range 60 −720 nM in PBS-BSA (4 g/L), as reported in Table 3. Aliquots of 100 μL of each were added to either 200 μL MeOH or 200 μL PBS in Eppendorf vials. All vials were centrifuged at 10,000 rpm (8600 g) for 5 min to separate the protein precipitate from the MeOH solution, whereas the PBS-BSA had no sediment. Aliquots of 50 μL of the MeOH supernatant or the PBS-BSA solution were added to 950 μL HUG solution (0.05 g/L) in PBS. Fluorescence was measured and the results are reported in Table 4.

The recovery data obtained show that BR quantification by HUG was the same whether the solution contained BSA or was deproteinized. Furthermore, data show that the presence of 3.3 % methanol does not interfere with the HUG assay.

In conclusion, the use of albumin-stabilised solutions ensures the reproducibility of the measurement. However, if it is necessary to work in the absence of protein, both BSA-based standard and sample deproteinization in methanol can be performed. In contrast, albumin-free aqueous solutions of BR are not suitable for absolute quantification, due to incomplete solubilization and time-dependent decay.

Limitations

In this protocol, only methanol was tested as a solvent for the extraction of BR from biological samples. Other solvents or their mixtures that could be used in certain laboratories were not tested here, since the main goal of this protocol is to guide the preparation of nanomolar bilirubin standards in physiological solutions.

Ethics statements

None

Supplementary material *and/or* additional information [OPTIONAL]

None

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Paola Sist: Conceptualization, Investigation, Methodology, Formal analysis, Visualization, Writing – original draft. **Federica Tramer:** Conceptualization, Investigation, Validation, Data curation, Formal analysis, Writing – review & editing. **Ranieri Urbani:** Conceptualization, Formal analysis, Writing – original draft. **Antonella Bandiera:** Resources, Writing – review & editing. **Sabina Passamonti:** Conceptualization, Methodology, Formal analysis, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Data availability

Data will be made available on request.

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