Protocol for the assay of biliverdin by the recombinant protein HUG

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Method Article

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Abstract

Biliverdin is a secondary metabolite of heme catabolism. It is formed by the reaction catalyzed by heme oxygenase, which converts the heme group contained in proteins, such as hemoglobin, myoglobin, cytochromes, and catalase, to biliverdin, iron (II), and CO in equimolar amounts, consuming NADPH. Biliverdin is then reduced to bilirubin by biliverdin reductase (again by the simultaneous oxidation of NADPH), which together forms an intracellular redox couple. Heme oxygenase-1 is an inducible enzyme induced by hypoxic conditions, increased availability of heme, or proinflammatory mechanisms, such as LPS, UV radiation, etc. Moreover, both heme oxygenase-1 and biliverdin reductase play roles other than catalysis, by modulating specific metabolic pathways at the transcriptional level. We present here a protocol for the determination of biliverdin based on its enzymatic conversion to bilirubin, which specifically binds to the recombinant protein HUG, resulting in fluorescence emission. This method allows the measurement of bilirubin and biliverdin in nM concentrations.

Introduction

Biliverdin (BV) is a green pigment produced by the breakdown of heme. It is formed by the enzyme heme oxygenase (HO), which cleaves the heme ring and produces biliverdin, carbon monoxide (CO), and free iron (Fe^{2+}) in equimolar amounts[1]. Subsequently, biliverdin is reduced to bilirubin (BR) by the enzyme biliverdin reductase (BVR)[2].

While in the past biliverdin was considered merely an intermediate in the formation of bilirubin, its antioxidant, anti-inflammatory, and cytoprotective roles are now recognized, particularly in the immune system[3-5]. Alterations in the expression and activity of HO and BVR have been associated with various pathological conditions[6-8] mediated by activation or inhibition of specific processes or metabolic pathways, such as cellular respiration or the production of proinflammatory cytokines. These effects can be due to increased cellular levels of BR and BV, which act as a redox couple with antioxidant properties[9,10].

In addition to this role, it is becoming increasingly clear that HO-1 exerts "non-canonical" protective functions due to its compartmentalization in the nucleus, where it plays a regulatory rather than a catalytic role[11,12]. In this respect, quantification of BR and/or BV within cells and in their extracellular medium could provide a direct approach to assess and distinguish canonical vs non-canonical functions, under experimental conditions that activate this metabolic pathway.

Highly sensitive methods for measuring nM concentrations of BV are based on HPLC, such as HPLC-TLS[13], BV-binding fluorescent proteins, such as smURFP[14] or iRPF[15], or fiber-enhanced Raman spectroscopy[16]. The only methods that simultaneously measure both BR and BV in the nM range are based on HPLC, coupled to mass spectrometry[17] or thermal lens spectrometry[13], but require a sample preparation step.

Here we present a protocol for the analysis of BV in biofluids or cell and tissue extracts, based on its enzymatic conversion to BR that is quantified fluorometrically by its specific binding to the recombinant protein HUG[18]. Given the highly selective interaction of BR with HUG, minimal sample volumes are required. This protocol can be seen as an add-on item to the protocol for the fluorometric analysis of BR in 96-multiwell plates[19].

Reagents

Ultrapure water milliQ for the preparation of all solutions

Dimethyl sulfoxide (DMSO) (Merck, SHBM2271)

Sodium hydroxide (NaOH) (Sigma-Aldrich)

Hydrochloric acid (HCl) (Sigma-Aldrich)

Acetone (Sigma-Aldrich)

Bovine Serum Albumin, Fraction V (BSA) (Merck, 12K1608)

Bilirubin (BR) (Merck, B4126)

Biliverdin (BV) (Merck, 30891)

Biliverdin reductase A human (BVR, 1500 U/mL) (Merck, B3687)

Dulbecco's Phosphate Buffered Saline (PBS) (Merck, SLCB9248)

NADPH tetrasodium salt (Merck, N7505)

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

Equipment

LABWARE

Laboratory tweezers

Stainless steel spoon

Beaker (0.5 L)

Laboratory glass bottles (1L)

1.5 mL vials (Sarstedt)

2 mL vials (Sarstedt)

15 mL-tubes (Sarstedt)

50 mL-tubes (Sarstedt)

Amber glass vials (10 mL)

Tips (10 mL, 1 mL, and 200 µL) (Sartstedt)

96-well black polystirene plates (Nunc®)

Gilson pipette 1000-5000 µL

Eppendorf Research plus pipette 500-1000 µL

Eppendorf Research plus pipette 20-200 µL

Eppendorf Research plus pipette 2-20 µL

Eppendorf Research plus pipette 0.5-10 µL

PTFE Magnetic Stirrer Bar

Quartz cuvette (l = 1 cm)

Lab Tube Racks

Aluminum foil

INSTRUMENTS

Multi-purpose water purification system (Crystal EX, Adrona®)

Microbalance (Mettler Toledo, XS205 Dual range)

Magnetic stirrer (Bibby Sterilin LTD®, UK)

pHmeter (pH Basic 20, CRISON, HACH®)

Spectrophotometer (Cary 4E, Varian)

Microplate reader (Synergy H1; BioTek Winooski, VT)

Thermostatic oven

Procedure

PRINCIPLES

Standard BV solutions are placed in a 96-well microplate, containing HUG. BV is converted to BR by adding the enzyme mix (BVR plus NADPH). Any BR formed will bind to HUG (Kd = 2.2 nM)[20], which drives the BVR reaction to completion. At the reaction steady state, fluorescence emission is measured in a microplate reader.

PREPARATION OF PBS and PBS-BSA SOLUTIONS

· *PBS pH 8.5*

Add 5 PBS tablets in 950 mL of ultrapure water.

Introduce a magnetic bar and mix on a magnetic stirrer.

Adjust the pH to 8.5 under the pH-meter by adding 1 M NaOH drop by drop.

Bring the volume to 1 L with Ultrapure water.

· *PBS-BSA SOLUTION*

Dissolve 0.2 g BSA powder in 50 mL PBS pH 8.5 in a tube to obtain a 4 $g \cdot L^{-1}$ BSA solution.

Introduce a magnetic bar and mix on a magnetic stirrer.

· *$PBS\text{-}BSA_{dil}$ SOLUTION*

Dilute 10 times the PBS-BSA solution in PBS, pH 8.5 to obtain a 0.4 $g \cdot L^{-1}$ BSA solution.

Introduce a magnetic bar and mix on a magnetic stirrer.

PREPARATION OF BILIRUBIN AND BILIVERDIN STANDARD SOLUTIONS

All solutions must be prepared in dim light. The BR and BV solutions must be prepared in amber glass vials or in aluminum-coated tubes (Table 1).

· *SOLUTION A*

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

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

Pipette the solutions into 20 µL aliquots in Eppendorf tubes and store at -20°C.

Stability: 4 months at -20°C.

· *SOLUTION B*

Dilute 10 µL *Solution A* containing either BR or BV in 4990 µL of PBS-BSA 4 $g \cdot L^{-1}$ and vortex. Wait 30 minutes and then prepare the 1 µM BR and BV solutions in PBS-BSA 4 $g \cdot L^{-1}$.

Stability: 4 days at 4°C for.

· *SOLUTION C*

Dilute serial volumes of *Solution B* in PBS-BSA_{dil} to a final volume of 1 mL.

The volumes are shown in Table 2.

Stability: stable at 4°C for 24 h.

PREPARATION OF CO-FACTOR AND ENZYME SOLUTIONS

· *NADPH stock solution*

Weigh a few mg of the NADPH powder in an Eppendorf tube on a precision microbalance.

Add an appropriate volume of PBS pH 8.5 to obtain a 10 mM solution and mix with vortex.

Aliquot 50 µL of these solutions in Eppendorf tubes and store at -20°C.

Stability: 4 months at -20°C.

· *Enzyme mix solution*

Dilute 20 µL of NADPH stock solution in a 2 mL Eppendorf tube with 1980 µL of PBS pH 8.5 to obtain a 0.1 mM solution of NADPH solution and shake.

Add 0.5 µL of BVR solution (1500 U/mL) to obtain a dilute working enzyme mix solution (0.375 U/mL BVR and 1 mM NADPH.

Stability: Use and analyze immediately after prepared.

QUALITY CONTROL OF BILIVERDIN AND BILIRUBIN STANDARD SOLUTIONS BY UV-VIS SPECTROSCOPY

Add 3 mL of *Solution B* to a quartz cuvette (d = 1 cm). Use PBS-BSA 4 $g \cdot L^{-1}$ as blank.

Record UV-VIS spectra in the range of: $300 < \lambda < 600$ nm.

Solution B should be considered accurate if the absorbance value is:

BR: 0.636 (± 0.05) at λ max = 470 nm

BV: 0.405 (± 0.03) at λ max = 380 nm

[BR] and [BV] can be calculated using the corresponding extinction coefficients (pH 8.5):

BR: e = 62408 ± 1062 cm^{-1} M^{-1}

BV*:* e = 39477 ± 1184 $cm^{-1}M^{-1}$.

EXECUTION OF THE HUG-BASED FLUOROMETRIC ASSAY OF BILIVERDIN STANDARD SOLUTIONS

In a 96-well black polystyrene plate, arrange 2 sectors (BR sector and BV sector), each made of 28 wells (4 columns x 7 rows). Each row will contain one of the serial concentrations of BR or BV (solutions C), each replicated in 4 wells (Figure 1).

Sequentially add the following solutions:

10 µL HUG (1 $mg \cdot mL^{-1}$) in each well of both sectors

100 µL of *PBS-BSA$_{dil}$ solution* in the BR sector

100 µL of *enzyme mix solution* in the BV sector (final enzyme mixture concentration 0.05 mM NADPH and 0.1875 $U \cdot mL^{-1}$ BVR).

100 µL *Solution C* containing 0, 2, 4, 20, 50, 80, 100 nM BR in the BR sector (fill 4 wells for each BR concentration)

100 µL *Solution C* containing 0, 2, 4, 20, 50, 80, 100 nM BV in the BV sector (fill 4 wells for each BV concentration)

Cover the plate and incubate at T = 25°C for 3h

Read the fluorescence in the multiplate reader (λ_{ex} = 485 nm, λ_{em} = 528 nm, T = 25 °C).

DATA ANALYSIS

Calculate the mean ± standard deviation (n=4) of fluorescence data resulting from each BR or BV (as BR equivalent, BR_{eq}) concentration.

Plot the mean fluorescence ± standard deviation vs [BR] or [BR_{eq}] (nM).

Fit the data of standard curves by linear regression analysis.

Troubleshooting

· Avoid direct light (work in subdue light and use amber glass vials).

· Reduce solutions exposure to air.

· Soak the amber glass vials leaving the vials overnight in aqueous NaOH solution (0.1 M); clean with aqueous HCl solution (0.1 M). Finally, wash several times with ultrapure water.

· Clean the quartz cuvettes with diluted DMSO or acetone and then wash them several times with ultrapure water.

· *Solution B* can be used for several days, but check its absorbance in the spectrophotometer to ensure that the concentration has not changed due to degradation or contamination before use.

· Incubate *Solution B* for at least for 30 min before reading the absorbance in the spectrophotometer to ensure that the solution reaches equilibrium.

· [BV]/[NADPH] ratio should be at least 1/5

· The reaction time may change depending on the type of samples to allow for a complete enzymatic reaction.

· It is possible to leave the plate overnight at 25°C in dim light when analyzing complex samples.

· Source of error or deviation are:

- exposure of BR and BV solutions to light;

- solution evaporation during the incubation period at T = 25°C;

- uncalibrated pipettes;

- inaccurate volume taken during dilution steps;

- the residual volume protruding from the tip can produce some concentration errors.

Time Taken

Preparation of enzyme mixture: 10 min

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

Incubation time for standard solution in PBS-BSA: 3 h 25°C

The overall time needed to complete the HUG-based fluorometric analysis: approximately 4 hours.

Anticipated Results

· Calibration curves of BR and BV (expressed as BR equivalents, after enzymatic conversion of biliverdin) give the regression parameters present in Table 3.

· By combining this assay with the BR standard protocol[19], both BR and BV can be quantified in various samples such as blood, cell extract and other biological fluids.

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Tables

Due to technical limitations, Tables 1-3 can be found in the Supplementary Files section.

Figures

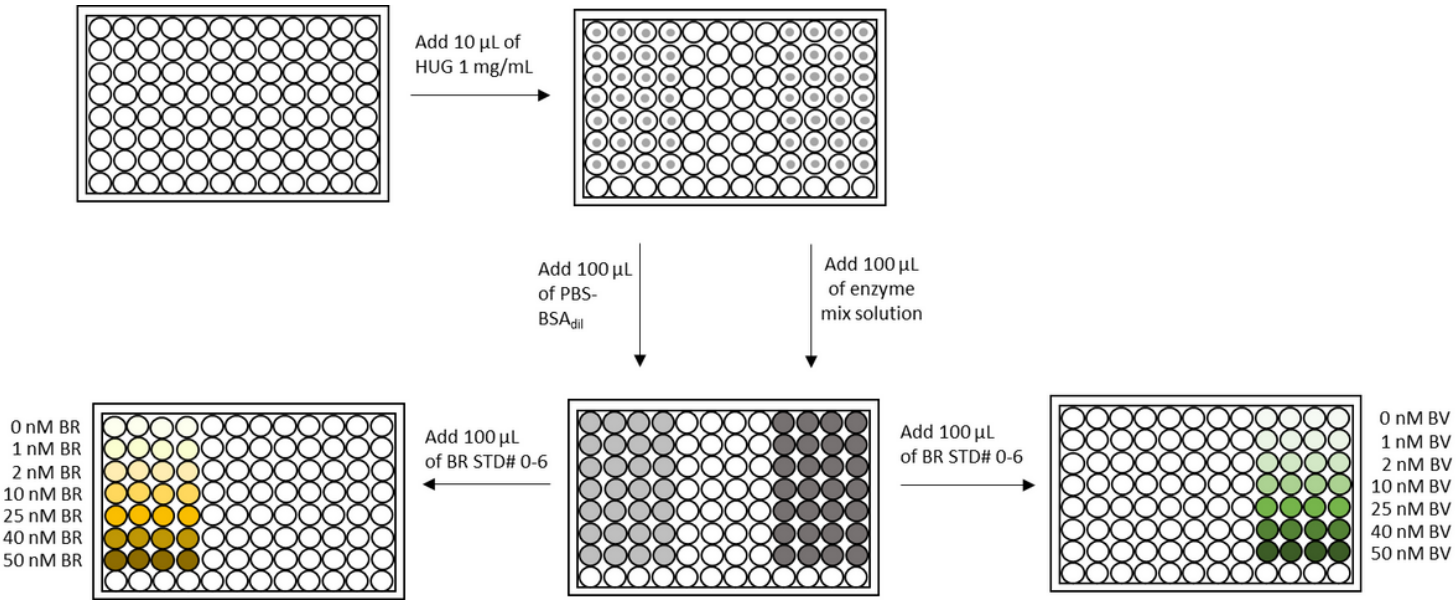

Figure 1

Scheme of the preparation of the 96-well plate. The standards final concentrations range is 0-50 nM.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table1.png
- Table2.png
- Table3.png