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Combined fluorometric analysis of biliverdin and bilirubin by the recombinant protein HUG ☆,☆☆

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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 into biliverdin, iron (II) and CO in equimolar amounts, consuming NADPH. Biliverdin is then reduced to bilirubin by biliverdin reductase. Biliverdin and bilirubin form a redox couple and are important for the redox homeostasis of cells. Heme oxygenase-1 is an inducible enzyme that is induced by hypoxic conditions, increased availability of heme or proinflammatory mechanisms such as LPS, UV radiation, etc. In addition, both heme oxygenase-1 and biliverdin reductase play roles other than catalysis by modulating specific metabolic pathways at the transcriptional level. There is a need for affordable assays to analyze these bile pigments in biological and clinical samples. Here we present a method for the combined determination of biliverdin and bilirubin that utilizes the specific binding of bilirubin to the fluorescent recombinant fusion protein HUG and the enzymatic conversion of biliverdin to bilirubin.

- This method enables the combined measurement of bilirubin and biliverdin in the nM range.
- The method does not require solvent extraction or protein precipitation of the samples.

Specifications table

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More specific subject area:	Bile pigment metabolism or Heme catabolism
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Background

Biliverdin (BV) is a green pigment produced by the breakdown of heme. It is formed by the enzyme heme oxygenase (HO, E.C.1.14.14.18), which cleaves the heme ring and produces biliverdin, carbon monoxide (CO) and free iron (Fe^{2+}) in equimolar amounts [1]. Biliverdin is reduced to bilirubin (BR) by the enzyme biliverdin reductase (BVR, E.C.1.3.1.24) [2]. Bilirubin and biliverdin form a redox couple that occurs both in cells and in the extracellular fluid. Both pigments act as antioxidant molecules and protect the cells from oxidative stress, which is mainly triggered by peroxyl radicals. Their interconversion depends on the activity of biliverdin reductase, which can reduce biliverdin back to bilirubin in the presence of NADPH [3]

While in the past biliverdin was considered merely an intermediate in the formation of bilirubin, its antioxidant [4,5], anti-inflammatory and cytoprotective role are now recognized, particularly in the immune system [6-8]. Alterations in the expression and activity of HO and BVR have been associated with various pathological conditions [9-11]. Therefore, quantification of bilirubin and biliverdin in serum, other human and animal biofluids, and in preclinical models may provide new insights into the dynamic status of heme catabolism in health and disease.

Highly sensitive methods for measuring nM concentrations of BV are based on HPLC, such as HPLC-TLS [12], BV-binding fluorescent proteins, such as smURFP [13], iRPF [14], fibre-enhanced Raman spectroscopy [15] or metal nanoclusters [16]. The only methods that simultaneously measure BR and BV in the low nM range are based on HPLC coupled with mass spectrometry [17,18] or thermal lens spectrometry [12], but require a sample preparation step.

Here we present an optimized method for the combined analysis of BV and BR in animal plasma, which may also be applicable to other biofluids. The assay utilizes the recombinant fusion protein HUG (HELP-UnaG) [19], in which the BR-specific fluorescent protein UnaG [20] is fused to a human elastin-like polypeptide (HELP). Due to its high affinity for BR [21], HUG selectively and efficiently extracts only BR and not conjugated BR from biological matrices, like UnaG [20]. The analysis of BV is performed by adding the enzyme BVR and its co-enzyme NADPH to the samples, so that BV is stoichiometrically converted to BR and the latter binds to HUG. The combined analysis of BV and BR is based on a set of optimized conditions for the analysis of BR at the nanoscale in plasma by HUG, as previously described [22]. This method to analyse BV as BR equivalents can be applied as complement of other methods that exploit UnaG, whether free or bound to surfaces or other macromolecules.

Method details

Materials

Analytical grade chemicals purchased from Merck were: Bilirubin (BR, purity 99 %), Bovine Serum Albumin fraction V (BSA, purity >98 %), Biliverdin (BV), Biliverdin reductase A human (BVR, 1500 IU/mL), Dulbecco's Phosphate Buffered Saline (PBS), NADPH tetrasodium salt reduced form, Dimethyl sulfoxide (DMSO), Sodium hydroxide (NaOH). Ultrapure water milliQ was used to prepare each solution. HUG was synthesized and purified as described [19]. A detailed protocol has been submitted (MEX-D-24–00,508).

Equipment

- Multi-purpose water purification system (Crystal EX, Androna®)
- Magnetic stirrer (Icamag® Rec-G)
- Analytical Balance ABT 120–4NM (KERN 770)
- Black 96-well plates (Nunc®, purchased by Thermofisher, code 237,107; polystyrene, sterile, non–treated surface)
- Synergy H1; BioTek, Winooski, VT, USA

Solutions

1. Phosphate Buffered Saline (PBS) pH 8.5: standard PBS solution was taken to pH 8.5 with NaOH 1 M.
2. Bovine Serum Albumin Solution (PBS-BSA): 4 g/L BSA in PBS pH 8.5.

   The solution can be kept at 4 °C for a couple of days. For longer storage, sterilization of the solution with 0.2 μm filter is advised.
3. BR and BV Stock Solutions: 5 mM in DMSO.

   The solution can be kept at −20 °C for at least 4 months.
4. BR and BV working solution: 10 μM BV or BR in PBS-BSA.

   Wait 30 min before use to allow the complete solubilization. Record UV–VIS spectra in the range of 300 < λ < 600 nm and use the BV and BR extinction coefficients at pH 8.5 (Table 1) to calculate the actual concentrations in the working solutions.

   The 10 μM solutions are sTable 4 days at 4 °C. Working solutions can be used for several days, but check their UV–VIS absorbance to verify that their concentrations are stable.
5. NADPH stock solution: 10 mM NADPH in PBS pH 8.5.

   Aliquots can be kept at −20 °C for at least 4 months.

Table 1
UV–Vis spectroscopic results and derived parameters of BV and BR solutions. Serially diluted solutions of BR and BV (1 – 10 μM) were prepared in PBS-BSA. Solutions were freshly prepared from the standard bilirubin stock and immediately analyzed by UV–VIS spectrometry in a transparent 96-well plate between 300 and 600 nm. A_{max} was recorded in a 10 μM BR solution after blank subtraction. All experiments were performed in triplicate adding 250 μL to each well.

	BR in PBS-BSA	BV in PBS-BSA
A_{max} (A.U.)	0.450±0.003	0.291±0.007
λ_{max} (nm)	465	380
optical pathlength (cm)	0.67	0.67
ε (cm−1 *M*−1)	66,980	43,369

6. Biliverdin reductase stock solution: 1.5 IU/μL BVR.
7. Enzyme mixture: 3.7 IU/mL BVR and 1 mM NADPH in PBS pH 8.5.
8. HUG stock solution: the recombinant protein HUG is not yet commercially available. It can be produced according to the previously described procedure [19]. The pure protein was dissolved in mQ water up to 2 mg/mL.

   Prepare the solution the day before to allow complete solubilization by dissolving the lyophilized HUG with stirring and then store at 4 °C for < 7 days. If necessary, it can be stored at −20 °C for at least 1 month.
9. HUG working solution: 0.05 mg/mL in PBS pH 8.5.

Procedure

In all solutions and samples preparation procedures, care should be taken to minimize exposure to light, whether artificial or natural light.

The combined analysis of both biliverdin and bilirubin by means of the so-called HUG assay [22] is based on the optimized BV conversion to BR, which is fluorometrically quantified.

The assay procedure is described by the protocol detailed as follows:

Preparation of BV and BR standard solutions

- Dilute the BR and BV working solutions (10 μM) to 1 μM in PBS-BSA.
- Dilute serial volumes of 1 μM BV or BR solutions in HUG working solution (0.05 mg/mL) to a final volume of 1 mL, as illustrated in Table 2.

All solutions must be prepared in tubes wrapped with aluminum foil under dim light.

Preparation of biological samples

Proper sample storage and preparation are essential for consistent and accurate results.

Prepare plasma/serum from animal blood using standard concentrations of heparin or EDTA as anticoagulants and standard protocols of centrifugation. Dilute murine or fish plasma/serum 100- or 10-fold in 0.05 mg/mL HUG PBS pH 8.5, respectively, immediately prior to use. For a triplicate determination of BV and BR in murine or fish plasma/serum, including a single aliquot for blank measurement, you need at least 15 or 140 μL of the sample, respectively.

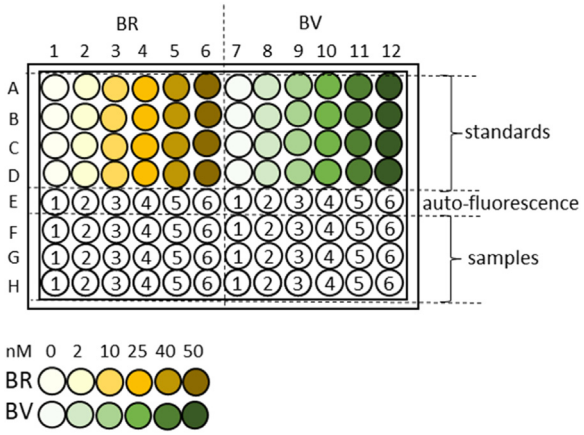
Dilute urine samples 25 to 100-fold in 0.05 mg/mL HUG in PBS pH 8.5. For a triplicate determination of BV and BR, including a single aliquot for blank measurement, you need at least 80 μL of urine. controllare protocollo

Biological samples must be stored at −80° to ensure BV and BR stability.

Considering that plasma/serum are diluted at least 25-fold, no interference from anti-coagulants has been observed.

Table 2
Scheme for the preparation of nM BV and BR solutions in the concentration range 2–50 nM.

Standard concentration (nM)	HUG 0.05 mg/mL (μL)	1 μM BR or BV (μL)
0	1000	–
2	998	2
5	995	5
10	990	10
25	975	25
50	950	50



Scheme 1. Typical 96-well calibration plate. The plate is loaded with BV and BR standard solutions. The remaining wells can be loaded with real samples. If you need to load a larger number of samples, it is not necessary to introduce a calibration curve for each plate.

Preparation of the 96-well plate

- Prepare the enzyme mixture (3.7 IU/mL BVR; 1 mM NADPH);
- Add 10 µl of enzyme mixture to each well of the BV sector of the 96-well plate, as shown in Scheme 1.
- Add 10 µl of PBS pH 8.5 to each well of the BR sector and to the autofluorescence wells of the 96-well plate, as shown in Scheme 1.

Execution of the assay

- Distribute 200 µL of each of the above described BV and BR standard solutions to pre-defined sectors of a 96-well black polystyrene plate, according to Scheme 1 (from well A1 to D6 for BR, from A7 to D12 for BV).
- Prepare samples with and without HUG as described in "*Combined analysis of BV and BR in mouse plasma and urine"* of the Method Validation section and add them to the plate (sample 1 E-H 1 and 7; sample 2 E-H 2 and 8 and so on as reported in the Scheme 1).
- Incubate the multiwell plate(s) at 25 °C in the dark for 3 h.

The time to the reaction completion may change, depending on the matrix of the biological samples. Overnight incubation ensures reaction completion.

- Read fluorescence intensity (λ_{ex} = 485 nm, λ_{em} = 528 nm; gain 100, reading height 2.50 mm; 25 °C) in a benchtop multiplate reader.

Data analysis

Calibration of the assay

- Subtract the blank fluorescence signal (corresponding to 0 nM standard solution) from the fluorescence signals of serially diluted standards (mean fluorescence of wells A-D 1 for BR and wells A-D 7 for BV).
- For each BV or BR concentration, calculate their fluorescence mean ± standard deviation (n = 4).
- Plot the mean fluorescence ± standard deviation vs [BV] or [BR] (nM). Fit the data by linear regression analysis to obtain the angular coefficient.

Bilirubin and Biliverdin data analysis in biological samples

- Subtract the autofluorescence signal (microwell E 1) to sample (microwells F-H 1) fluorescence values for BR and calculate the mean value (F_{BR}), then subtract the autofluorescence signal (microwell E 7) to sample (microwells F-H 7) fluorescence values and calculate the mean (F_{TBR}).
- Determine the net fluorescence related to BV concentration (F_{BV}) as follows:

$$F_{BV} = F_{TBR} - F_{BR}$$

where

F_{BR} is the fluorescence signal in the absence of BVR and NADPH + H^+, and refers to BR.

F_{TBR} is the fluorescence signal in the presence of BVR and NADPH + H^+, and refers to the sum of BR + BV (said "total BR" or TBR).

- Calculate BR and BV concentrations using the angular coefficient of the calibration curve

Statistical analyses

Data were analyzed and plotted using GraphPad Prism 10.1.0 (264) (GraphPad Softwares).

Method optimization under standard conditions

In order to determine the optimal assay conditions in terms of pH, temperature, cofactors and BVR concentration, we measured the fluorescence performance over the entire BV concentration range (0, 10, 25 and 50 nM) to avoid a possible bias due to the use of a single BV concentration. The fluorescence intensity at each BV concentration was fitted by linear regression analysis and the angular coefficient (nM^{-1}) was determined. Optimal experimental conditions were considered to be achieved when the maximum angular coefficient was reached in the time course analysis of the above parameters. All reactions were tested in the dark at 25 °C for up to 16 h, unless otherwise stated.

Progress of the BVR reaction with either NADH or NADPH

The aim of the first optimization step was to determine the time required to complete the conversion of BV to BR in the presence of NADPH or NADH added in a molar excess (10 μM) over BV. This assay was performed either at a pH of 7.4 (the intrinsic pH of plasma and serum) or at a pH of 8.5, the pH optimum for the NADPH-dependent reaction [23,24].

The microtiter plate was prepared as described in the "Execution of the assay" section, but the nanomolar standard solutions of BV and BR were diluted in PBS-BSA at either pH 7.4 or pH 8.5. Fig. 1 shows the NADPH- or NADH-dependent reaction course at pH 7.4 and pH 8.5.

At a pH of 7.4, BR formation from BV in the presence of NADH was slower than in the presence of NADPH, but reached the same steady state. In contrast, at pH 8.5, only the NADPH-dependent reaction reached steady-state, while the NADH-dependent reaction was still incomplete.

The reaction was completed in < 1 hour under standard conditions with NADPH as coenzyme in the pH range 7.4–8.5, whereas with NADH the reaction was only completed after at least 3 h. The fluorescence signal was stable for up to 16 h after reaching steady state, which is probably due to the BR stabilization property of HUG [22]. Since this method can be performed under different conditions and in different biological matrices, the preferential use of NADPH as a coenzyme of the BVR reaction is suggested.

The dependence of the BVR reaction on the coenzyme concentration

The aim of this optimization step was to determine the optimum coenzyme concentration at the most favorable pH values, i.e. pH 7.4 for NADH and pH 8.5 for NADPH.

Based on the data, the lowest coenzyme concentration required for the complete conversion of BV to BR in the range of the experimental BV standard concentration could be determined. As shown in Fig. 2, the minimum NADPH coenzyme concentration was 5 μM, while NADH was 50 μM. This 10-fold difference has already been found in several studies characterizing the enzymology of BVR [23,25].

The dependence of the BVR reaction on the temperature

Temperature is an important variable in enzyme kinetics. In addition, other assay variables are also influenced by temperature, e.g. the solubility of HUG, which undergoes a phase transition at 42 °C [21], leading to its aggregation, the evaporation of the assay buffer and the possible growth of bacteria in the wells. In addition, the conversion of BV to BR in some matrices may take longer than for standard BV solutions. We measured the effects of two temperatures (25 °C and 37 °C) on the rates of NADPH- and NADH-

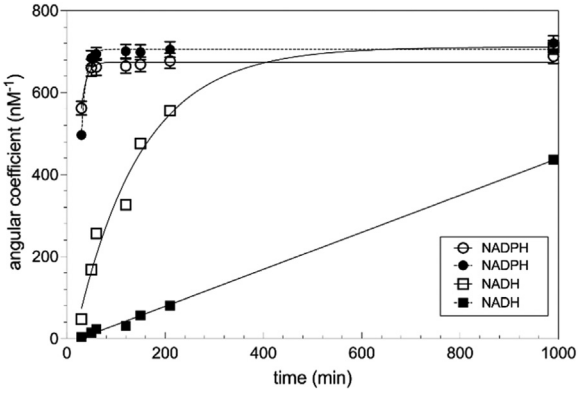


Fig. 1. Progress of the BVR reaction with either NADH or NADPH. The variation of the angular coefficient (obtained plotting the BR and BV concentrations vs fluorescence) over time at 25 °C is shown at pH 7.4 (empty symbols) and pH 8.5 (solid symbols) by using 10 μM NADPH (circles) or NADH (squares). Data were fitted to the equation: $y = y_M(1 - e^{-kx})$, where y_M is the maximum reached value (697 ± 20), k is the rate constant (min^{-1}): 0.09546 with NADPH at pH 7.4; 0.1095 with NADPH at pH 8.5; 0.007551 with NADH at pH 7.4. Data obtained with NADH at pH 8.5 were fitted to the equation $y = ax + c$, where $a = 0.4513$, $c = -11{,}53$. Some error bars are shorter than the size of the symbol.

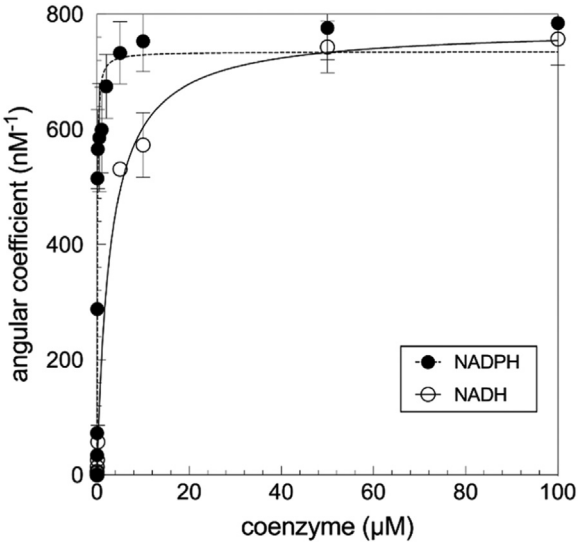


Fig. 2. The dependence of the BVR reaction on the coenzyme concentration. Variation of angular coefficient (obtained plotting the BV concentrations vs fluorescence) as a function of increasing coenzyme concentrations. The reaction took place in the presence of increasing concentration (0–100 μM) of NADPH at pH 8.5 (solid symbols) or NADH at pH 7.4 (open symbols) using 0.1875 IU/mL BVR. Data were fitted to the equation $y = ax/k + x$ where a is the highest angular coefficient obtained in these experimental conditions, k is the concentration of coenzyme that gave half maximal angular coefficient. The parameters obtained at 25 ˚C were $k = 0.0760$ and $a = 735.1$ for NADPH (dotted line); $k = 2.854$ and $a = 775.7$ for NADH (continuous line).

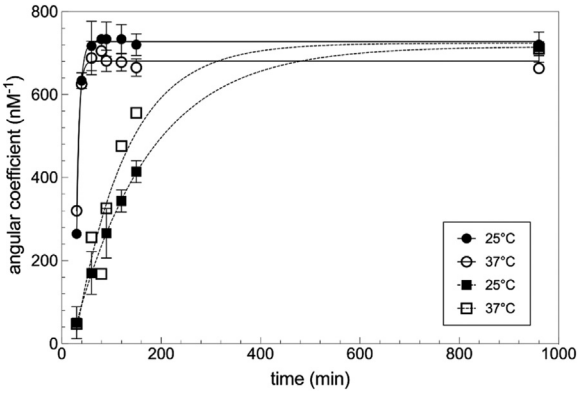


Fig. 3. The dependence of the BVR reaction on the temperature. The variation of the angular coefficient in nM^{-1} (obtained plotting the BV concentrations vs fluorescence) over time is represented at 25 ˚C (solid symbols) and at 37 ˚C (open symbols). Data were fitted to the equation: $y = y_M(1 - e^{-kx})$ where y_M is the maximum reached value, k is the rate constant (min^{-1}); $k = 0.158$ at 25 ˚C; $k = 0.189$ at 37 ˚C with 0.1 mM NADPH (circles and continuous line); $k = 0.00652$ at 25 ˚C; $k = 0.00977$ at 37 ˚C with 0.1 mM NADH (squares and dotted line).

dependent reactions at pH 8.5 and 7.4, respectively. The data show (Fig. 3) that both the NADPH- and NADH-dependent reactions proceeded at similar rates at 25 ˚C and 37 ˚C and reached similar steady-state values. The ratio of the reaction rate constant k_{NADPH}/k_{NADH} was 24 at 25 ˚C and 19 at 37 ˚C, respectively, demonstrating the stronger dependence of BVR on NADPH. Based on these results, 25 ˚C was chosen as the standard experimental condition.

The dependence of the BVR reaction on the enzyme concentration

The aim of this test was to determine the optimum enzyme concentration required to convert BV (in the range of 0–50 nM) with the lowest amount of enzyme. The progress of the reactions in the presence of different concentrations of BVR was recorded for up to 16 h (Fig. 4A), and the calculated rate constants at each enzyme concentration were plotted against [BVR] (Fig. 4B), showing that the optimal BVR concentration was ≥ 0.185 IU/mL.

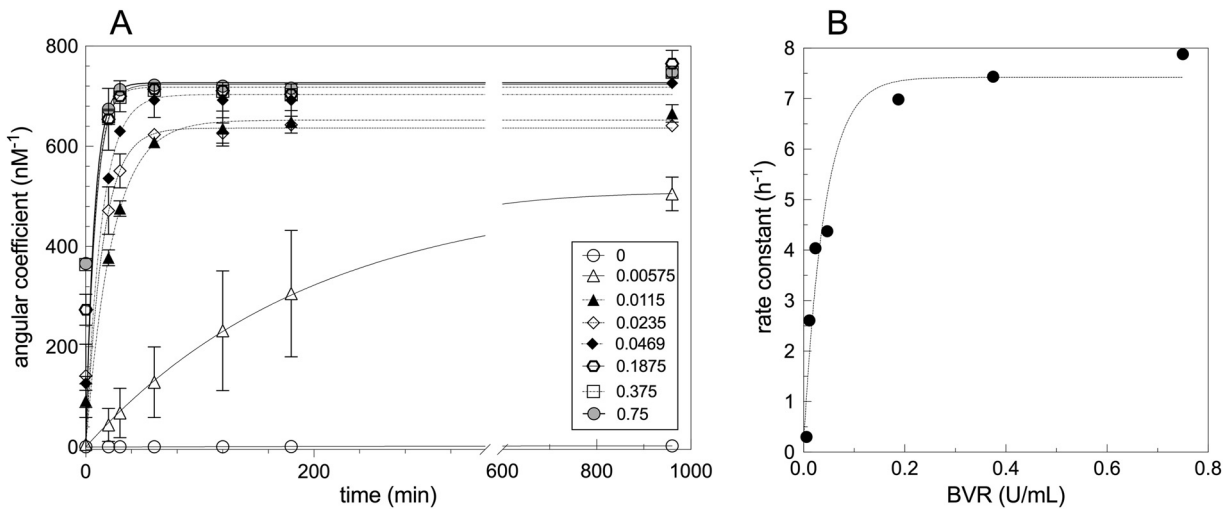


Fig. 4. The dependence of the BVR reaction on the enzyme concentration. (A) kinetics of angular coefficient variation at different BVR concentrations. After 16 h of incubation, data show that the optimal BVR concentration was at least 0.185 U/mL. Data were fitted to the equation: $y = y_M(1 - e^{-kx})$, where y_M is the maximum reached value; k is the rate constant (h^{-1}); (B) the rate constant of reaction at each BVR concentration.

Method validation

To validate our method, we first generated a series of BR and BV calibration curves in BSA to evaluate the complete conversion of BV to BR. We then determined the recovery of BR and BV in plasma and urine of wild-type and Bvra $^{-/-}$ mice and finally determined the physiological BR and BV concentrations in the plasma of the same animals.

Assessment of full conversion of bv to BR

The assay was validated by assessing the capacity of this assay to convert BV to BR in a predicted 1:1 stoichiometry.
Based on the above tests, the assay optimal conditions were chosen as follows:

PBS pH 8.5,
[NADPH] = 0.05 mM,
[BVR] = 0.185 IU/ml
T = 25 °C
Reaction time = 3 h (or overnight for biological samples).

Under our assay conditions some biological samples must be diluted so not to exceed a total bile pigments concentration of 80 nM.

Serial dilutions of BR and BV standard solutions in the range of 0.5 – 50 nM were distributed in a 96-well plate as shown in Scheme 1. The fluorescence emitted from the two standard solutions was fitted by a linear regression equation. The angular coefficients obtained from multiple experiments (n = 19 for BR; n = 18 for BV) were compared using the unpaired t-test, and no significant difference was found (p = 0.116). Thus, under the identified test conditions, BV was converted to BR in a 1:1 stoichiometry, as expected.

Since the angular coefficients of the BV and BR calibration curves showed no statistically significant difference, all fluorescence data were fitted by linear regression analysis to obtain a single angular coefficient (748 ± 9) for both pigments (Fig. 5).

Combined analysis of BR and BV in mouse or fish plasma

This assay was performed with real samples consisting of plasma and urine from 9-month-old mice of two strains, the WT and its double knock-out strain Bvra $^{-/-}$ (both C57Bl/6 genetic background), which is unable to physiologically reduce BV to BR [26] and therefore exhibits abnormal biliverdinemia. Furthermore, this assay was implemented on seebass (*Dicentrarchus labrax*) plasma, whose predominant bile pigment is biliverdin.

Assessment of BR and BV recovery in murine plasma and urine

To determine a possible matrix effect, we performed a spike-and-recovery experiment. Known volumes (5 µL) of BR or BV standard solutions (0.5–8 µM) were added to the plasma (10 µL) and urine (20 µL) of the two animals to increase the basal pigments to known levels in the low, medium and high nM range. The same procedure was performed with a reference solution of PBS-BSA, which has

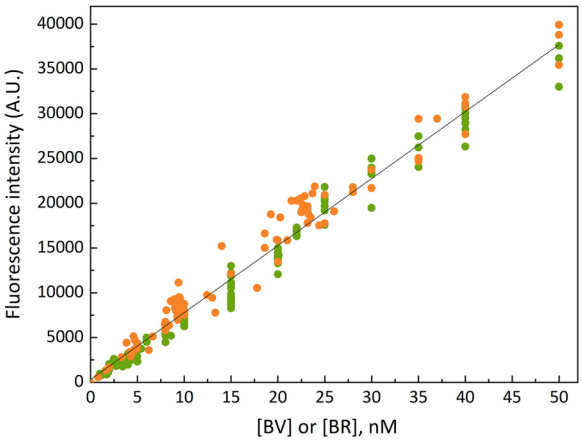


Fig. 5. Analysis of both bilirubin and biliverdin by HUG. Analysis of BV and BR was performed as described in the text. Fluorescence data from individual calibration tests ($n = 19$ with BV, green circles; $n = 18$ with BR, orange circles) were combined and fitted to the linear equation $y = a + b^*x$. The parameters obtained were: $a = 305 \pm 177$; $b = 748 \pm 9$; $R^2 = 0.97$.

Table 3
Recovery of bile pigments in the mouse plasma and urine under spiking conditions. Results were analyzed by 2-way ANOVA with Šindák's multiple comparison test using standard significance level $\alpha = 0.05$.a

Sample (n)	Spike Level, nM	Expected **BSA**	Observed **WT**	Recovery %	*p*-value	Observed ***Bvra*** $^{-/-}$	Recovery %	*p*-value
BR Plasma ($n = 2$)*	Low	3.8 (±0.1)	4.1 (±0.3)	108	*0.974*	4.58 (±0.09)	111	*0.838*
	Med	20.1 (±0.3)	19.7 (±0.1)	98	*0.954*	19.6 (±0.1)	97	*0.997*
	High	38.3 (±2.0)	38.7 (±1.9)	101	*0.831*	37.5 (±4.4)	98	*0.999*
BV Plasma ($n = 2$)*	Low	3.6 (±0.1)	4.1 (±0.3)	86	*0.946*	3.6 (±0.1)	81	*>0.99*
	Med	18.75 (±0.2)	21.59 (±0.1)	87	*0.195*	21.3 (±0.3)	95	*0.262*
	High	40.69 (±3.2)	39.63 (±1.9)	92	*0.781*	35.8 (±4.4)	98	*0.897*
BR Urine ($n = 3$)**	Low	3.65 (±0.3)	4.1 (±0.2)	112	*0.300*	3.96 (±0.4)	108	*0.430*
	Med	16.4 (±0.3)	16.30 (±0.4)	99	*0.933*	16.59(±0.4)	101	*0.800*
	High	32.77(±0.7)	32.62 (±0.7)	99	*0.857*	32.84 (±0.7)	100	*0.967*
BV Urine ($n = 3$)**	Low	3.7 (±0.1)	3.3 (±0.1)	90	*0.716*	3.5 (±0.4)	96	*0.919*
	Med	15.7 (±0.1)	15.9 (±0.2)	101	*0.919*	16.7 (±0.8)	106	*0.149*
	High	31.8 (±0.2)	31.10 (±2.8)	98	*0.513*	32.17 (±1.4)	101	*0.273*

* n represents the number of animals with which the analyzes were carried out in triplicate.
** n represents the number of replicates performed on a pooled urine sample from different animals. Results were analyzed by 2-way ANOVA with Šídák's multiple comparison test using standard significance level $\alpha = 0.05$.

no matrix effect. After diluting the plasma, urine and BSA reference solutions 100- and 50-fold, respectively, in HUG working solution with or without biliverdin reductase mix, the samples were spread in black 96-well plates (3 replicates) and incubated at 25 °C for 16 h. The BV and BR concentrations in the spiked plasma and urine samples and their estimated recoveries are shown in Table 3.

The recovery in plasma and urine of wild type and in *Bvra* $^{-/-}$ mice showed no significant differences between the observed and expected values. The low standard deviation shows the good reproducibility of the test.

Combined analysis of BV and BR in mouse plasma and urine

The following protocol was performed for the combined analysis of BR and BV in plasma and urine of wild type and Bvra $^{-/-}$ mice:

1. Dilute 20 µL of mouse plasma and 40 µL of mouse urine in 2 mL of HUG working solution (0.05 mg/mL in PBS pH 8.5).
2. Dilute 5 µL mouse plasma or 10 µL mouse urine in 0.5 mL PBS pH 8.5 for autofluorescence of the sample
3. Add 10 µL PBS pH 8.5 for BR analysis and autofluorescence uptake to the multiwells as shown in Scheme 1 (microwell A1-H6)
4. Add 10 µL enzyme mixture (3.7 IU/mL BVR, 1 mM NADPH, in PBS pH 8.5) for BV analysis as shown in Scheme 1 (microwell A6-H12).
5. Add 200 µL of diluted plasma or urine to the multiwell plate in both the BR and BV sectors (at least in triplicate for example sample 1 in columns 1 and 7 rows F-H).
6. Add 200 µL of the autofluorescence sample to a well (sample 1 in wells D1 and D7)
7. Incubate the 96-well plate overnight at 25 °C.
8. Read the fluorescence.

Table 4
Basal BR and BV concentration in wild type and Bvra $^{-}$ mouse plasma and urine from our and already published data.

	BR (nM) (mean ± sem)	BV (nM) (mean ± sem)	Ref.
plasma			
Wild type (n = 2)*	559 ± 118	25 ± 10	Our data
Bvra $^{-/-}$ (n = 2)*	40 ± 10	182 ± 33	
Wild type	1449 ± 1035	99 ± 22	[26]
Bvra $^{-/-}$	1 ± 2	4 ± 2	
Wild type	1175 ± 181	4 ± 1	[27]
Bvra $^{-/-}$	9 ± 2	100 ± 30	
Wild type	1680 ± 270	44 ± 7	[28]
Bvra $^{-/-}$	< 36	146 ± 15	
urine			
wt (n = 3)**	43 ± 14	82 ± 4	Our data
Bvra $^{-/-}$ (n = 3)**	< 1	4040 ± 56	
Wild type	28 ± 37	116 ± 34	[26]
Bvra $^{-/-}$	21 ± 4	5742 ± 4016	

* n represents the number of animals with which the analyzes were carried out in triplicate.
** n represents the number of replicates performed on a pooled urine samples from 6 animals.

Table 5
Recovery of bile pigments in the fish plasma under spiking conditions.

Sample (n)	Spike Level	Expected **BSA**	Observed **fish**	Recovery %	p-value
BR Plasma (n = 2)*	Low	2.21 (± 0.16)	2.26 (± 0.12)	102	*0.990*
	Med	11.76 (± 0.04)	11.14 (± 0.13)	95	*0.015*
	High	23.06 (± 0.07)	22.90 (± 0.48)	99	*0.777*
BV Plasma (n = 2)*	Low	2.20 (± 0.03)	2.17 (± 0.28)	99	*0.999*
	Med	10.66 (± 0.02)	10.80 (± 0.07)	101	*0.902*
	High	22.16 (± 0.59)	22.24 (± 0.09)	100	*0.980*

* n represents the number of replicates performed on a pooled plasma sample from different animals.

Fluorescence data were analyzed as reported in “Data analysis” paragraph by using the calibration curve shown in Fig. 5. The results are shown in Table 4.

The data show that the wild-type mouse has a measurable plasma concentration of 40 nM BV, while the Bvra $^{-/-}$ mice have a 7-fold higher BV concentration in plasma. The BV data obtained with the HUG methods are consistent with published data (see ref. in the table), while our BR values are lower, especially in the wild-type mouse. The BR/BV ratio of 22 in the wild-type mouse was reversed in the Bvra $^{-/-}$ mouse, where the BV concentration is 4.5-fold higher than that of BR.

Urine bile pigment values are consistent with published data for both strains.

In summary, our assay is ideal for dosing very small amounts of pigment in standard solutions and biological samples. Due to the high affinity of the HUG, the pigments do not need to be extracted with organic solvents and the BR and BV content can be determined simultaneously.

Assessment of BR and BV recovery in fish plasma

We performed a spike-and-recovery experiment in seebass plasma. Known volumes (5 µL) of BR or BV standard solutions (0.5–8 µM) were added to 70 µL plasma. As above, the same procedure was performed with a reference solution of PBS-BSA, which has no matrix effect. After diluting the plasma and BSA reference solutions 10 fold in HUG working solution with or without biliverdin reductase mix, the samples were spread in black 96-well plates (3 replicates) and incubated at 25 °C for 16 h. The BV and BR concentrations in the spiked plasma and urine samples and their estimated recoveries are shown in Table 5.

Also in fish plasma, complete BR and BV recovery was achieved. The 95 % recovery observed with medium level of BR is regarded as a random finding with negligible impact on the measurement accuracy.

Limitations

This assay provides an indirect measurement of the BV concentration and always requires the simultaneous determination of bilirubin. Incubation times can vary greatly depending on the matrix in which these analytes are measured and must be optimized accordingly.

One limitation is that the BV and BR standards occupy half plate, leaving free wells for only 6 samples. However, not every plate has to have the standard curve, as mentioned above. A number of standard-free plates, each containing up to 12 different samples and

their respective auto-fluorescence blanks, can be associated to a single standard plate. In facts, the incubation time is long enough for full and stable conversion of BV to BR in a relatively large set of plates incubated overnight.

CRediT author statement

Federica Tramer: conceptualization, resources, investigation, methodology, validation, data curation, formal analysis, writing–original draft, visualization. **Paola Sist:** investigation, formal analysis, writing–review & editing. **Rocio Cardenas-Perez:** investigation, validation, data curation. **Ranieri Urbani:** conceptualization, formal analysis, writing–original draft. **Giulia Bortolussi:** animal work, review & editing. **Sabina Passamonti:** conceptualization, methodology, formal analysis, writing–review & editing, supervision, project administration, funding acquisition.

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.

Ethics statements

Mice were housed and handled according to the institutional guidelines. Ethical and experimental procedures were reviewed and approved by the ICGEB board, with full respect to the EU Directive 2010/63/EU for animal experimentation, and by the Italian Ministry of Health (Project 523/2017-PR). All experiments involving animals were conducted in full respect of the ARRIVE 3R principles. Mice were kept in a temperature-controlled environment with a 12/12 h light/dark cycle. They received a standard chow diet and water ad libitum. Male wild type animals used in this study were from C57/Bl6 genetic background. Male Bvra$^{-/-}$ mice with a C57/Bl6 background were generated as previously described [27]. Fish plasma was a kind gift of the fish farm Friskina Ltd. Croatia.

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

None.

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