Application of high-performance liquid chromatography combined with ultra-sensitive thermal lens spectrometric detection for simultaneous biliverdin and bilirubin assessment at trace levels in human serum

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ABSTRACT

We present the applicability of a new ultra-sensitive analytical method for the simultaneous determination of biliverdin and bilirubin in human serum. The method comprises isocratic reversed-phase (RP) C18 high-performance liquid chromatography (HPLC) and thermal lens spectrometric detection (TLS) based on excitation by a krypton laser emission line at 407 nm. This method enables the separation of IX-α biliverdin and IX-α bilirubin in 11 min with limit of detection (LOD) and limit of quantitation (LOQ) for biliverdin of 1.2 nM and 3 nM, and 1 nM and 2.8 nM for bilirubin, respectively. In addition, a step-gradient elution was set up, by changing the mobile phase composition, in order to further enhance the sensitivity for bilirubin determination with LOD and LOQ of 0.5 nM and 1.5 nM, respectively. In parallel, an isocratic HPLC-DAD method was developed for benchmarking against HPLC-TLS methods. The LOD and LOQ for biliverdin were 6 nM and 18 nM, and 2.5 nM and 8 nM for bilirubin, respectively. Additionally, both isocratic methods were applied for measuring biliverdin and free bilirubin in human serum samples (from 2 male and 2 female healthy donors). Combining isocratic HPLC method with TLS detector was crucial for first ever biliverdin determination in serum together with simultaneous free bilirubin determination. We showed for the first time the concentration ratio of free bilirubin versus unbound biliverdin in human serum samples.

1. Introduction

Biliverdin belongs to the family of tetrapyrrolic bile pigments, and is derived in vertebrates from heme catabolism. In the first step, the cleavage of the α-methene bridge in heme to form biliverdin IX-α, iron and carbon monoxide is catalyzed by heme oxygenases. Subsequently, biliverdin IX-α is reduced to bilirubin IX-α by biliverdin reductase (BVR) [1], as shown in Supplementary material. Beside the bioactive IX-α isomers, there are also structural (e.g. III-α and XIII-α) and geometric (ZZ, EE, ZE, EZ) isomers of both biliverdin and bilirubin. Biliverdin has been correlated with numerous health protective actions, namely i) anti-inflammatory activity by inhibiting the activation of NF-kappaB [2,3]; ii) anti-ischemic activity, e.g. reduction of cerebral infarction [4]; iii) protective for organ preservation in organ grafts, e.g. for liver, renal, and cardiac transplantations [5]; iv) protective against insulin-resistance [6]; v) antiviral actions, e.g. reducing replication of hepatitis C virus [7]; vi) protective role against ethanol-induced gastric damage [8]; vii) protective against diabetic nephropathy [9]; vii) lately a study has proven that patients with Parkinson's disease had higher values of biliverdin, and their bilirubin/biliverdin ratio were decreased [10]. This shows the importance of following bilirubin/biliverdin ratio as a biomarker of oxidative stress-related diseases.

Biliverdin protective activity can be explained via its anti-inflammatory, anti-oxidant, anti-apoptotic, anti-proliferative, and immunomodulatory properties. Biliverdin undergoes fast conversion via biliverdin reductase (BVR) into bilirubin, which is the most potent physiological antioxidant. It is speculated that bilirubin is re-cycled back to biliverdin upon the exposure to oxidative agents. This fast redox cycle involving BVR has attracted much attention as a catalytic sink for reactive oxygen (ROS) and nitrogen species (RONS) in the intracellular compartment [11,12]. BVR activity in cells is important under conditions such as hypoxia and inflammation in healthy tissue [1]. Interestingly, also cancer cells survival seems to be linked to the HO-biliverdin-BVR pathway,

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protecting them against oxidative stress and chemotherapy [13,14].

Despite the fact that these bile pigments are widely present in biological samples, the presence of biliverdin has not been proven in vivo during normal human heme metabolism, except possibly in trace amounts, demonstrating that its reduction by BVR is prompt and quantitative. To add to the analytical challenge, biliverdin interacts with serum albumin [15] and serum alpha(1)-Acid glycoprotein [16]. The stoichiometry for these interactions is unknown, due to analytical bottlenecks.

Analyses of bilirubin and biliverdin have been mainly performed by HPLC using reversed-phase (RP) columns in combination with UV/vis detection [18–23] as well as in combination with mass spectrometry (MS) using electro spray ionization (ESI) source [24,25]. As mentioned before, metabolomic technologies using HPLC-ESI-MS/MS were applied for the identification and relative quantification of novel biomarkers for Parkinson's disease including biliverdin and bilirubin [26,27]. Electrochemical methods [28–32], capillary electrophoresis with laser-induced fluorescence detection [33], simultaneous injection effective mixing analysis system using spectrophotometry [34] and chemiluminescence via bilirubin and peroxynitrite reaction were also used for bilirubin determination [35], while for biliverdin determination there are no other analytical methods reported. All in all, the afore-mentioned methods were used mainly for total bilirubin and/or biliverdin determination in biological samples present in amounts above 10 nM. Moreover, no published method is available, which would enable quantitation of unbound biliverdin in serum samples, and only a few methods have been developed for free bilirubin determination in serum, namely i) a well-known indirect determination using peroxidase test [36]; ii) indirect and specific spectrofluorimetric quantification of bilirubin in serum samples with LOD of 4.8 nM by using yttrium–norfloxacin complex as a fluorescence probe [32]; iii) and only lately, by combining HPLC with TLS detection, a first ever direct free bilirubin determination in human and animal serum samples was successfully performed with LOD and LOQ of 90 pM and 250 pM, respectively [23]. Therefore, it is clear why there are no data available on (unbound) biliverdin concentration levels in serum.

The fact that there is no direct measurement available that could provide data on biliverdin concentration in any animal serum sample could be explained by the lack of proper sample preparation and lack of selective and sensitive analytical method. For instance, simultaneous analysis of biliverdin and bilirubin was performed only in pericardial fluid using a HPLC-UV/vis method with LOQ for both pigments above 100 nM [20]. Similarly, the determination of biliverdin together with other biliverdin related compounds was accomplished in rat liver using HPLC-UV and HPLC-ESI-MS/MS [20] and the determination of biliverdin together with porphyrins was achieved in bile and excreta of birds by HPLC-UV [22]. Additionally, fluorescence spectroscopy was used to evaluate the interaction between biliverdin and bovine serum albumin [37].

All methods published to date lack selectivity and sensitivity for simultaneous quantification of both unbound (free) biliverdin and (free) bilirubin in biological samples. Yet, these would be powerful tools for the discovery of relevant clinical biomarkers of redox-based diseases.

It was therefore, our objective to develop a novel method for simultaneous determination of both bilirubin and biliverdin in biological fluids by coupling HPLC to ultra-highly sensitive TLS detection [38], which we present here. The applicability of the method was ensured by a tested sample preparation of human serum sample [23].

2. Experimental

2.1. Chemicals and standards

HPLC grade methanol (MeOH) and synthetic grade acetic acid (AcOH) were purchased from Sigma-Aldrich (Steinheim, Germany). HPLC analysed dimethyl sulfoxide (DMSO) was purchased from J.T. Baker (Deventer, The Netherlands). Ammonium acetate (NH_4OAc) was purchased from Merck (Darmstadt, Germany). TLC grade biliverdin hydrochloride ($\geq$ 97%) and bilirubin ($\geq$ 99%) were purchased from Applichem (Darmstadt, Germany); both were stored at -20 °C.

Individual standard solutions of bilirubin and biliverdin were prepared as follows: an accurately weighed amount of standard (2–3 mg) was dissolved in appropriate volume of DMSO to gain 10 mM solution. Subsequently, dilutions till 10 μM concentration were made by separately pouring 1 mL aliquots of each individual standard solution into 10 mL flat-bottomed volumetric flask, which was then filled up to 10 mL with MeOH. Further dilutions were made using a mixture of MeOH:H_2O (3:1, v/v). Twice deionized water was used. All solutions were prepared fresh daily and the whole sample preparation workflow was performed in dim light.

2.2. HPLC-DAD analyses

HPLC-DAD analyses were carried out using Agilent 1100 Series system (Agilent Technologies, Waldbronn, Germany) fitted with a thermostated autosampler (G1313A ALS) with a 500 μL loop, a quaternary pump (G1311A QUAT PUMP), degasser (G1322A) and with a diode-array detector (G1315A DAD). The evaluation of the collected data was made by ChemStation software (for LC 3D system Rev. B01.03 204, Agilent Technologies 2001–2005). Injection volume for each run was 200 μL. Flow rate was set to 0.6 mL/min, the column temperature was set to 25 °C and the acquisition wavelengths were 377 nm, 407 nm and 457 nm. Bilirubin and biliverdin were separated using a stainless steel BDS Hypersil C18 column (100 mm × 4.6 mm I.D.) with a pre-column (4.0 mm × 4.6 mm I.D.) with particle size of 2.4 μm and pore size of 120 Å (Thermo Fisher Scientific, Waltham, USA). The isocratic elution was made using 34% of 20 mM NH_4OAc solution in MeOH.

2.3. HPLC-TLS analyses

HPLC-TLS analyses were performed using P200 Binary Gradient Pump (Thermo Separation Products – Thermo Scientific, Waltham, MA, USA), a manual injector (Rheodyne, Model 7725) with a 200 μL loop, and a dual-beam TLS detection unit with a 8 μL flow-through cell with 1 cm optical path length, which are schematically presented in Fig. 1. The HPLC column is described in Section 2.2. The isocratic elution was made using 34% of 20 mM NH_4OAc water solution (pH of 6.95 and not corrected) in MeOH, while the step gradient elution was performed using buffers A and B composed of MeOH: 100 mM NH_4OAc: AcOH (72: 27: 1, $v/v/v$) and MeOH:AcOH (98:2, v/v), respectively. The following step gradient was applied: 100% A (0–4 min), 100–0% A (4–5 min), 100% B (5–15 min). Simultaneous bilirubin and biliverdin detection and quantification was accomplished using the TLS experimental setup consisting of a Krypton laser (Coherent, Innova 300C, Santa Clara, CA, USA) with excitation beam tuned to 407 nm providing 115 mW of power. A helium-neon (He-Ne) laser (Melles Griot, Uniphase, model 1103P, Carlsbad, CA, USA) provided the probe beam at 632.8 nm with 2 mW of power. The changes in probe-beam intensity after passing the flow-through cell were monitored behind a pinhole by a pin photodiode equipped with a 633 nm interference filter (MELLES GRIOT) and connected to a lock-in amplifier

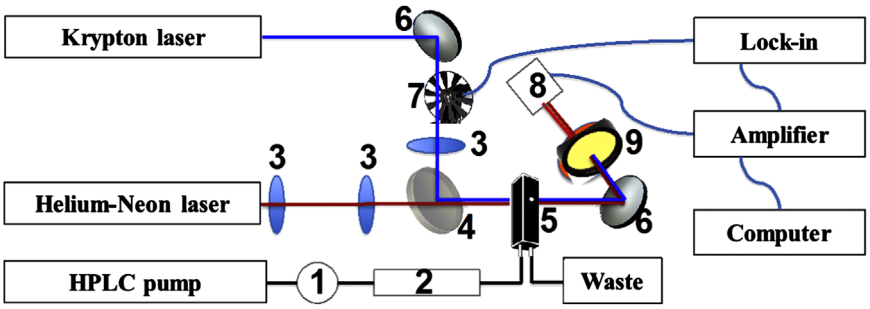

Fig. 1. Schematic presentation of HPLC-TLS system assembly: 1 – manual injector, 2 – HPLC column, 3 – lens; 4 – dichroic mirror; 5 – flow-through cell; 6 – mirror; 7 – chopper; 8 – photodiode and 9 – cut-off filter.

Table 1
Comparison between validation parameters of HPLC-DAD and HPLC-TLS methods.

Method	Isocratic HPLC-TLS			Isocratic HPLC-DAD		
Compound	bilirubin		biliverdin	bilirubin		biliverdin
Wavelength (nm)	406.7	[a]457.9	406.7	457	[a]457	377
Linearity range (nM)	2.5–250	0.25–100	2.5–250	7–1000	5.5–1000	10–500
[b]Slope (a)	0.0027	0.0029	0.0018	8.9	9.4	8.1
[b]Intercept (b)	−0.0009	−0.0005	−0,0012	−7.8	−11.5	−8.2
R^2	0.9991	0.9998	0.9990	0.9991	0.9998	0.9992
LOD (nM)	1.0 ([c]0.5)	0.09	1.2 ([c]1.2)	2.5	1.8	6
LOQ (nM)	2.8 ([c]1.5)	0.25	3.0 ([c]3.0)	8	5.5	1
System precision (RSD – % using standard solutions of 25 nM)	1.5	1.2	1.9	1.0	0.9	1.0
Repeatability between the sample replicates (RSD – %)	5.5	5.1	10.0	[d]n.d. < LOD	[d]n.d. < LOD	[d]n.d. < LOD

[a] Information obtained from Ref. [23].
[b] The equation y=ax+b states for curve, where y is the peak area, x the concentration of analyte (nM), a is the slope and b is the interception.
[c] LOD and LOQ using a step gradient HPLC-TLS method.
[d] Could not be determined, because the concentration is below LOD.

(Stanford Research Instruments, Model SR830 DSP, Sunnyvale, CA, USA), which amplifies only the component of the input signal that emerges with the frequency of the reference signal from mechanical chopper (Scitec Instruments, Redruth, Cornwall, UK). This is achieved by a Fourier transformation of the signal while filtering out all other frequencies with a low-pass filter. The lock-in amplifier is connected to a computer, where the data were collected using a Turbo Pascal program. The pump beam was modulated at a frequency of 28 Hz by a mechanical chopper. The applied frequency provides maximal signal to noise ratio (S/N) in a frequency range accessible by the applied chopper. Each beam was focused using a lens with 100 mm focal distance (Edmund Optics, Barrington, NJ, USA), while collinear propagation of focused pump and probe beams through the flow-through cell was achieved by a dichroic mirror (Melles Griot). The flow-through cell was located at the focal point of the excitation beam, whereas the probe beam focal point was located $\sqrt{3} \times Zc$ from the position of the excitation beam waist. Zc is Rayleigh length ($Zc=\pi \times a^2/\lambda$, where a is the probe beam radius in the waist and λ its wavelength). Its optimal position with respect to the focus of the probe beam was determined experimentally. The excitation beam powers were measured at the location of the flow-through detection cell.

2.4. Biliverdin and bilirubin quantification

The sample peaks representing biliverdin and bilirubin were identified on the basis of their retention time (t_R) values obtained by HPLC-TLS analysis and were compared to the t_R values obtained in the analysis of a bilirubin and biliverdin standard solution. The identity of both peaks was also confirmed by spiking the sample solution with a bilirubin and biliverdin standard solution. For the target compounds linear regression analyses were performed by the external standard method. Accordingly, the external standard calibration curves were generated daily using eight data points. The calibration curves in both HPLC-TLS and HPLC-DAD systems were obtained by plotting the peak compound area (μVs and mAUs, respectively) at each level versus the concentration (nM) of the sample.

2.5. Sample preparation

Blood samples were obtained from healthy volunteer donors aged 25–38 (two males and two females), and collected into red top Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ, USA). The blood serum was obtained as described in previous study and the fresh serum samples were immediately ultra-centrifugated at 36 °C using Amicon ultra – 0.5 mL centrifugal filter devices (Merck Millipore, Darmstadt, Germany). Two sequential centrifugation cycles were made as described in our previous study [23]. The volume of ultrafiltrate solutions was twice diluted with MeOH: H_2O (1:3, *v*/*v*). The analyses of four human serum samples were done in triplicates. Precision and repeatability in Table 1 are referred to peak areas representing bilirubin or biliverdin.

3. Results and discussion

3.1. HPLC assay

In order to explore the presence of unbound (free) biliverdin and free bilirubin in serum samples three basic requirements have to be met simultaneously: i) selectivity, in order to separate both compounds and avoid interference of other compounds present in serum sample, ii) sensitivity, for detection in trace concentrations, and iii) proper sample preparation.

Accordingly, a rapid baseline separation of investigated compounds was achieved using C18-RP HPLC column with particle size of 2.4 μm, which enabled using normal HPLC conditions, such as

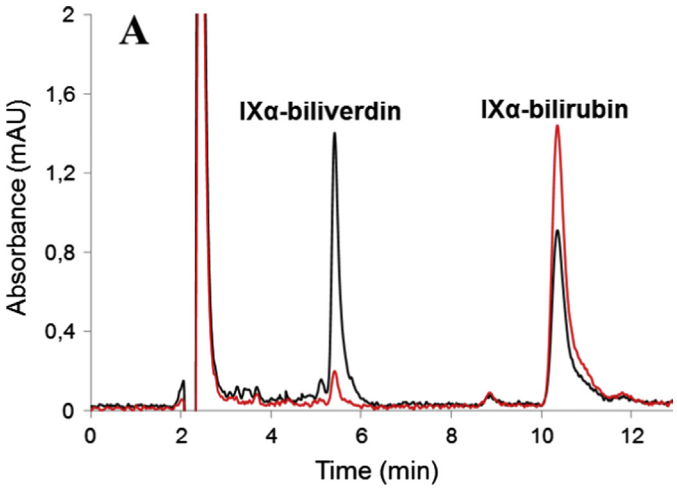

Fig. 2. (A) Comparison of HPLC-DAD chromatograms of IXα-biliverdin and IXα-bilirubin (both 100 nM) obtained by using detection wavelengths of 407 nm (black line) and 457 nm (red line). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

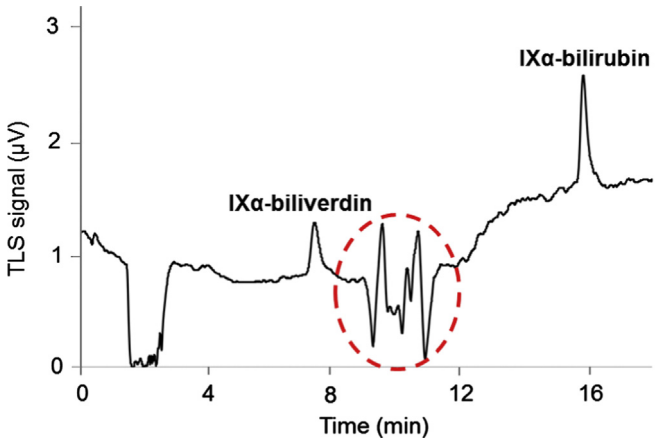

Fig. 3. HPLC-TLS chromatogram of separated Xα-biliverdin and IXα-bilirubin using step gradient elution and detection wavelength of 407 nm. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

sustainable pressure up to 250 bars and a standard 8 μL flow-through cell.

The Krypton laser, operating at irradiation power of 100 mV or more, provides emission at two wavelengths, i.e. λ=406.7 nm and λ=413.1 nm, which are sufficiently close to the maximal absorption wavelengths of both bilirubin and biliverdin (λ=457 nm and 377 nm, respectively). We have chosen for excitation the 406.7 nm line, which is closer to the absorption maximum of biliverdin since we wanted to achieve highest sensitivity for this analyte.

Separation of biliverdin and bilirubin is shown in Fig. 2. DAD-based detection was assessed at λ=407 nm, i.e. the laser emission line, or at λ=457 nm, i.e. the bilirubin maximal absorption. The chromatograms show that λ=407 nm enabled simultaneous detection of both biliverdin and bilirubin (Fig. 2).

The water content in mobile phase is also an important factor affecting TLS sensitivity due to direct dependence of TLS signal on thermo-optical properties of the medium in which the detection is performed [38]. In fact, the dependence on thermo-optical properties of solvents (i.e. temperature coefficient of refractive index ($\partial n/\partial T$), thermal conductivity (k)) makes TLS technique even more sensitive in comparison to convectional transmission mode absorption techniques. Water is however not the best solvent for TLS due to relatively small change in refractive index with temperature, and relatively high thermal conductivity, which decreases the TLS signal. Thus, we developed two different HPLC methods by varying between isocratic and step gradient elution. The goal was to achieve highest possible sensitivity of both methods, by changing the organic solvent (high $\partial n/\partial T$ and low k) and water (low $\partial n/\partial T$ and high k) content in the mobile phases. Indeed, the maximal thermal lens effect is achieved by maximizing $\partial n/\partial T$ while minimizing k [38]. The addition of NH_4OAc into all pH neutral used mobile phases as a counter ion (NH_4^+) to biliverdin and bilirubin was obligatory, otherwise none of injected compounds was detected. This is probable due to their elution in solvent front due to no retention, as the pKa values of biliverdin are 3.9 and 5.3, and of bilirubin are 4.2 and 4.9 [39]. In the case of bilirubin the water content in the mobile phase was minimized (from 34% to 2%) by the addition of AcOH without losing retention on the column when a step gradient elution was applied in HPLC-TLS (Fig. 3). However, a drawback of TLS detection technique when coupled with HPLC using gradient elution is that the base line noise is seriously compromised. This is due to the change in solvent composition, which results in a change and inhomogeneity of the refractive index that distorts the probe beam and deteriorates the TLS signal [40]. This effect on TLS signal is highlighted with red dashed-line circle in Fig. 3, where the noise enhancement due to applied gradient can be seen over a 3 min time frame, between retention times of 10 min and 13 min. When a step gradient elution was applied immediately after the elution of biliverdin and several minutes before the elution of bilirubin, LOD for bilirubin was approximately 0.5 nM. This is higher with respect to LOD of 90 pM reported in our previous study, which focused only on detection of bilirubin [23]. Higher LOD is due to the following factors: firstly, to lower absorbance of bilirubin at the applied excitation wavelength (28,500 $M^{-1}cm^{-1}$ at 407 nm vs. 54,400 $M^{-1}cm^{-1}$ at 458 nm) [41]; secondly, to longer retention time (t_R) of bilirubin (t_R of 16 min in step gradient mode versus t_R of 6 min in isocratic mode [23]), which results in about two-fold increase in half-peak width and in associated additional 2.3-times decrease in peak height as observed experimentally for equivalent concentrations in compared elution modes; finally, in agreement with our previous observations [40] soon after the completion of the gradient the TLS signal noise is still higher as compared to isocratic mode (by 25% for our step mode case). All this accounts for a 5,6 fold increase in LOD, which is in good agreement with the observed increase in LOD from 0.09 to 0.5 nM.

For the reasons discussed above no linear gradient elution was tested, since it would give raise to longer time of analysis, higher baseline noise, as well as to a sloping base line at the t_R of bilirubin, due to increasing TLS enhancement factor associated with the increasing % of the organic solvent in the mobile phase.

To avoid gradient elution and therefore to eliminate the baseline noise originating from the step gradient elution and to shorten the analysis time, we used the isocratic HPLC-TLS method with the same chromatographic parameters as in above-mentioned HPLC-DAD method, i.e. 34% water in MeOH and free of acetic acid. The absence of acetic acid affected the retention of both analytes, which was exhibited in about 40% decreased retention time for biliverdin as well as for bilirubin. The increase of water content (34%) in mobile phase enabled the separation of both bile pigments in less than 12 min, while maintaining the LOD for bilirubin unchanged, due to less signal noise, despite lower TLS enhancement factor (higher % of water in the mobile phase).

3.2. TLS assay and its comparison with DAD

The LODs and LOQs from either HPLC-TLS or HPLC-DAD are presented in Table 1. The achieved LOD and LOQ values for HPLC-

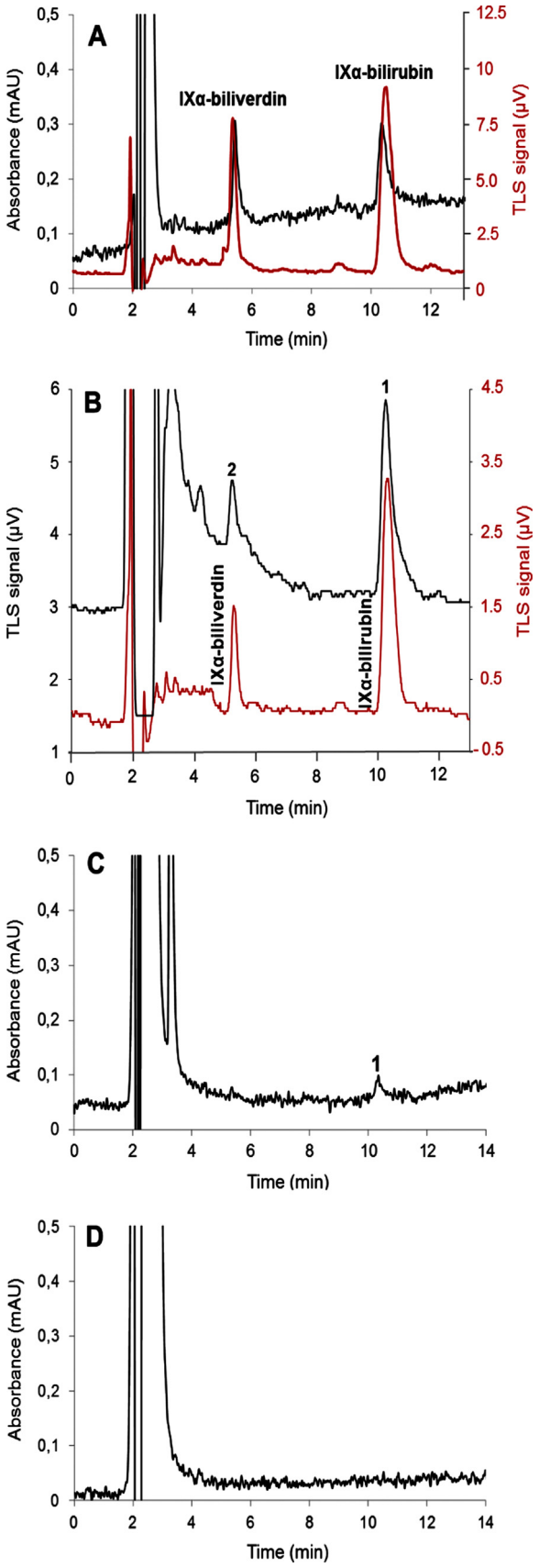

Fig. 4. (A) Comparison between HPLC-DAD (black line) and HPLC-TLS (red line) analysis of mixed standard solution of bilirubin and biliverdin (both 15 nM) detected at 407 nm; (B) Comparison between HPLC-TLS analysis of mixed standard solution of bilirubin and biliverdin (both 5 nM) (red line) and serum sample (black line) detection at 406,7 nm; (C) HPLC-DAD chromatogram of analysed serum sample detected at 457 nm; (D) HPLC-DAD chromatogram of analysed serum sample detected at 377 nm. All analyses were made by using isocratic elution (34% of water in MeOH). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

TLS method operated with the chosen 406.7 nm emission line were still lower than for HPLC-DAD, where detection at two wavelengths was used to match maximal absorptions of biliverdin and bilirubin (Table 1). The superior sensitivity of TLS with respect to DAD is presented in Fig. 4A. A standard mixture solution of 15 nM is analysed by the HPLC-DAD method at approximately its LOQ level (black line), while TLS detection gave a peak signal approximately 6-fold higher than its LOQ (red line). If DAD-based detection at 377 nm and 457 nm was used, to detect either biliverdin or bilirubin at their maximum absorptions, the LOQ and LOD values were approximately 5-times higher compared to HPLC-TLS method (Table 1). Higher sensitivity for bilirubin than for biliverdin was observed (Table 1), which could be partly associated with higher molar.

absortivity at 406.7 nm ($\approx 24{,}000\ M^{-1}\,cm^{-1}$ for biliverdin dimethyl ester [42] and $\approx 28{,}000\ M^{-1}\,cm^{-1}$ for bilirubin [40]) and to major part with higher TLS enhancement factor provided by mobile phase with higher % of organic solvent, i.e. the medium in which bilirubin is detected. Despite the fact that by using isocratic instead of gradient elution the base line noise is reduced, the main drawback of simultaneous biliverdin and bilirubin determination by using isocratic HPLC-TLS method is the lower sensitivity for bilirubin (as shown in Table 1), when compared with our previously developed HPLC-TLS method for bilirubin determination, which features considerably lower water content of the mobile phase (3% of water in methanol), and consequently a higher thermal lens enhancement factor.

However, the presented HPLC-TLS method is still sensitive enough to provide accurate measurements of free bilirubin while enabling simultaneous detection of biliverdin in serum samples as well. The obtained LOD and LOQ for biliverdin determination using HPLC-TLS are the lowest among methods published to date. To the best of our knowledge, so far only the HPLC-TLS method enabled the determination of biliverdin together with free bilirubin in serum sample as shown in Fig. 4B. With HPLC-DAD method we were only able to detect bilirubin at LOD values, but no biliverdin, at 457 nm (Fig. 4C) and neither biliverdin nor bilirubin at 377 nm (Fig. 4D).

3.3. Application to real samples

The isocratic HPLC-TLS method was applied for analysis of four human serum samples, without sample pre-concentration. The concentration levels of both unbound biliverdin and bilirubin and the respective bilirubin/biliverdin ratio are presented in Table 2.

This is the first-ever quantification of unbound biliverdin in a human serum sample. The relative standard deviation (RSD) of biliverdin determination was higher than for free bilirubin, because of its low physiological concentration level. However, it was still in the range of the method's LOQ (Table 1). The ratio between free bilirubin and unbound biliverdin content ranged 2.5–4.5. The only data available from literature on the bilirubin/biliverdin ratio is based on UPLC/MS/MS and concerns total bilirubin and total biliverdin in serum samples obtained from control group and from persons who had Parkinson's disease. The reported ratios are

Table 2
Free bilirubin and biliverdin concentrations and their ratios in human serum samples from the studied subjects. All results are presented as means +/− SD.

Serum samples	1	2	3	4
Sex	M	F	F	M
Age	24	25	38	35
Serum free bilirubin (nM)	9.0 ± 0.5	5.0 ± 0.3	12.0 ± 0.6	10.1 ± 0.2
Serum unbound biliverdin (nM)	2 ± 0.5	2 ± 0.5	2.8 ± 0.3	3 ± 0.3
Bilirubin/biliverdin ratio	4.5	2.5	4.3	3.4

1.5 and 0.8, respectively [27], but no further conclusions can be made about the agreement with the free bilirubin/unbound biliverdin ratio reported herein, since the formation constants of various bound biliverdin species are not known. Clearly, the bioactive species are those unbound, reliably measured by our method.

This immediately opens possibilities for novel applications of the presented method since its capability of simultaneous determination of biliverdin and bilirubin serum concentrations, offers the opportunity to assess the dynamic equilibrium of bilirubin and biliverdin in human serum under various (patho)physiological conditions. Another potential application is associated with the possibility for using exogenous biliverdin supplements to improve the antioxidant status, since they can be absorbed across the intestinal epithelium in vivo [16]. However, we need precise and accurate analytical methods to study biliverdin pharmacokinetics, i.e. absorption, distribution, metabolism, and elimination. Higher serum concentrations of both biliverdin and bilirubin also mean improved antioxidant and anti-inflammatory status of the person. This opens the potential to use those concentrations as biomarkers of healthy lifestyle – similar biomarker approach was recently suggested in asymptomatic middle-aged men for serum bilirubin levels, which are inversely correlated with the severity of metabolic syndrome [17].

From a physiological perspective, simultaneous assessment of biliverdin and bilirubin in the biological fluid will enable us to understand the controversial role of BVR in “redox-cycle” hypothesis. It is difficult to measure enzyme kinetics of BVR since it is a very fast process, however the newly developed method will enable us to follow the BVR enzyme kinetics in vivo and in vitro. In addition, there are numerous pharmacological approaches how to increase the serum bilirubin levels, e.g. inducing heme oxygenase-1 (HO-1) and BVR, activation of BVR, and inhibition of UDP-glucuronosyltransferase (UGT1A1). Thus, this method can be applicable also in nonclinical development to follow the concentrations of both, bilirubin and biliverdin, and thus assess the drug efficacy and activity.

Potential applications include also the indirect measurement of ROS activity in the intracellular compartment as bilirubin converts to biliverdin under ROS condition. Moreover, red blood cell (RBC) turnover rate could be measured, since biliverdin concentration could be influenced by the ratio of RBC degradation/RBC production rate (in certain pathologies). It was also found that the concentration ratio between total bilirubin and total biliverdin in serum samples was significantly lower in patients with Parkinson's disease than in the controls [27], which is in accordance with the fact that biliverdin is generated by ROS reactions with bilirubin [1].

4. Conclusions

We have established new frontiers for ultra-highly sensitive simultaneous analysis of biliverdin and bilirubin. Both developed isocratic methods (HPLC-DAD and HPLC-TLS) can be used for accurate and sensitive analysis of both pigments. However, when ultra-high sensitivity is needed, the TLS is the method of choice for detection as it was shown in the case of biliverdin determination in serum samples. The HPLC-TLS method itself excels in ultra-high sensitivity, by exploiting the TLS detection features combined with prompt and high resolving HPLC base line separations, which offers LODs, lower than 2 nM for both pigments. All this enables unbound biliverdin and/or free bilirubin determination in the concentration ranges lower than those accessible by previously available analytical methods (below 100 nM).

Our study revealed for the first time the presence of trace amounts of unbound biliverdin in human serum. Moreover, we determined the bilirubin/biliverdin concentration ratio of approximately 3 in the serum of 4 healthy volunteers.

Our method offers the opportunity of elucidating the bilirubin-biliverdin redox loop in serum, as well as in other biological fluids, and its correlation with various physiological and clinical conditions.

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