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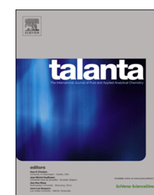

Application of high-performance liquid chromatography combined with ultra-sensitive thermal lens spectrometric detection for simultaneous biliverdin and bilirubin assessment at trace levels in human serum

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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.

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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

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