



# ANTIOXIDATIVE BLOOD STATUS ASSESSMENT BY THE FREE BILIRUBIN ASSAY

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**Abstract** — Bilirubin is an endogenous antioxidant in the human blood. While measurement of a total serum bilirubin, comprising of protein-bound (indirect bilirubin) and bilirubin glucuronide (direct bilirubin), is a routine clinical test, direct determination of free bilirubin represents an analytical challenge. Free bilirubin, which is the bioactive form, accounts for a minor fraction (<100 nM). Here we show the direct analysis of free bilirubin in human and animal blood serum samples using a state-of-the-art system comprised of newly developed high-performance liquid chromatography (HPLC) on reversed-phase (RP) C18 support coupled with thermal lens spectrometric detection (TLS), based on excitation wavelength of 457.9 nm using argon laser. The method excels in ultra-high sensitivity with limit of detection (LOD) and limit of quantitation (LOQ) of 90 pM and 250 pM, respectively. As a result, we present here the direct assay of free bilirubin in serum (around 10 nM), which can be employed as an assessment of antioxidative blood status.

**Index Terms** — TRANS2CARE, antioxidant, bilirubin, blood, HPLC, DAD, TLS

## 1 BACKGROUND

In clinical chemistry terms, serum bilirubin is classified as either indirect or direct. Elevated direct or indirect bilirubin levels (supra-physiological amounts) serve as diagnostic markers for liver and blood disorders. High serum bilirubin levels (> 200  $\mu\text{M}$ ) are neurotoxic in neonatal development, while bilirubin in lower concentrations acts as a strong antioxidant. Indirect serum bilirubin (5.0 - 17.0  $\mu\text{M}$ ) is unconjugated and thus it is poorly soluble in physiological solutions. It occurs as a reversible complex with serum albumin, and in the equilibrium with free bilirubin (< 100 nM).

Serum free bilirubin is gaining interest in preventive medicine, because higher serum bilirubin levels are correlated with the reduced cardiovascular disease risk. Thus, measuring free bilirubin in serum might become a predictive biomarker for the health status of the general population before the onset of clinical symptoms.

## 2 OBJECTIVES

Lack of analytical procedure for free bilirubin determination in serum is the main reason for the current conundrum on the applicability of the serum parameter's predictive value. Therefore our objectives are:

- To develop a HPLC-TLS method that could accomplish the first-ever direct determination of free bilirubin in blood serum
- To ensure that the HPLC-TLS method could be applied in clinical laboratories for research use and for blood analysis on daily basis
- To develop a method incorporating HPLC coupled with diode-array detector (DAD) for assessing higher bilirubin concentration levels ( $> 20$  nM). HPLC-DAD system is already present as standard equipment in clinical laboratories, thereby enabling immediate application without additional investment into existing infrastructure.

## 3 APPROACH & METHODS

### General approach

1. Blood collection. 2. Serum preparation. 3. Serum ultracentrifugation. 4. Free bilirubin analysis in ultrafiltrate solution.

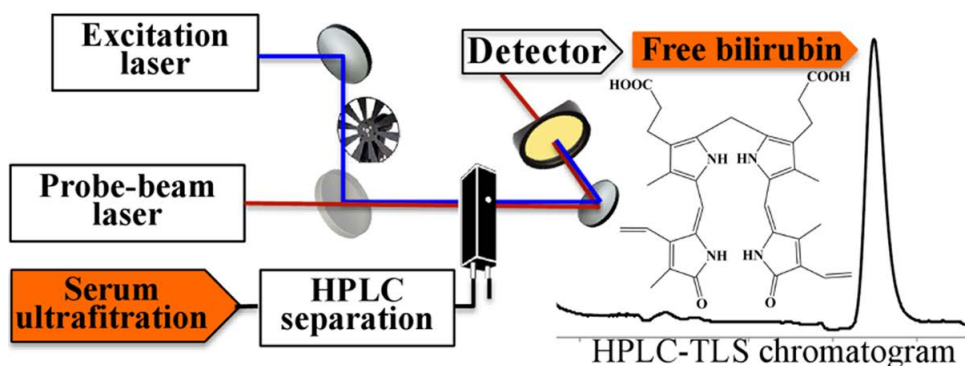
### Methods

Two possible methods for assessment of free bilirubin - by HPLC-TLS or HPLC-DAD methods were used, as recently published [1]. Brief graphical summary of the method is shown in Figure 1.

Figure 1: Schematic presentation of ultra-highly sensitive HPLC-TLS system for the determination of free bilirubin in serum samples. Bilirubin limits of detection and quantification are 90 pM and 250 pM, respectively

## 4 RESULTS

By the application of published HPLC-TLS method [1] the determination of free bilirubin is no longer allusive as shown in Figure 2. This opens new frontiers in clinical and pre-clinical investigations of bilirubin. It must be pointed out that the lack of appropriate free bilirubin analysis has so far prevented a full understanding of its physiology.



The study revealed that free bilirubin concentrations of healthy adults are around 10 nM, which is in the range of previously reported values obtained either theoretically or experimentally. The intrinsic value of this study method is that it allows a direct detection of bilirubin degradation products even at trace levels, which can be used as an indication of the quality of sample preparation procedure, so its accuracy with respect to other approaches increases.

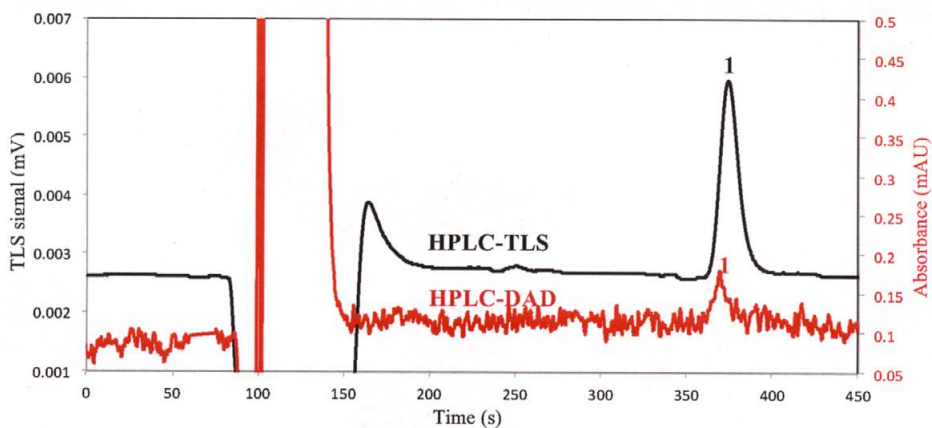
It should be taken in consideration that the HPLC-DAD method developed in this work could be easily applied in clinical medicine for free bilirubin determination in patients having elevated bilirubin levels such as encountered in the case of mild hyperbilirubinemia which does not require the ultra-high sensitivity provided by TLS [1].

Performed validation of both methods together with the sample preparation procedure meets criteria for their application in clinical medicine.

Figure 2: Comparison between HPLC-TLS (black line) and HPLC-DAD (red line) analysis of fresh human serum sample. Peak 1 represents free bilirubin signal. Evidently, TLS detection has 20-fold higher sensitivity when compared to DAD detection

## 5 POTENTIAL NEW PRODUCTS & SERVICES

Product: Measuring free bilirubin in biological samples (including serum samples) using the HPLC TLS system could become a standard practice in clinical laboratories interested in translational research. We can carry out studies showing the correlation between serum free bilirubin and a given i) physiological condition, such as pregnancy, physical training, fasting, eating or drinking, among others; as well as ii) pathological condition (any disease).



Service: accurate and fast free bilirubin analysis in serum samples can be utilized as service for customers such as medical institutions, scientific institutions, SMEs, which would like to do a given study (and get a given product), as well as to general public, which enables highly-motivated individuals to check their blood antioxidant status.

## 6 CURRENT COLLABORATIONS

### 6.1 With other researchers

The Universities of Nova Gorica (PP3, Trans2Care), Trieste (LP, Trans2Care), Ca'Foscari Venice (PP7, Trans2Care), Primorska (PP12, Trans2Care) and the General Hospital of Sempeter (PP6, Trans2Care) form a research consortium on bilirubin in biomedical research.

## 7 CONTACT OR COLLABORATIONS NEEDED

Future collaboration with clinical laboratories is needed.

## 8 COMMUNICATION TOOLS

- The high level of expertise in the field of analytical chemistry as well as the high level of applicability, quality and performance of the presented methods is disseminated through the high quality scientific publications.
- This method has been presented to the biomedical community at the University of Primorska (<http://www.trans2care.eu/NewsData.aspx?IdNews=68&ViewType=Old&IdType=390>), University of Ferrara (<http://www.trans2care.eu/NewsData.aspx?IdNews=81&ViewType=Old&IdType=390>), NanotechItaly (<http://www.trans2care.eu/NewsData.aspx?IdNews=90&ViewType=Old&IdType=390>), Slovenian Chemical Day on 12-14 September 2012 (Portorož, Slovenia) and Chemistry towards Biology on 10-13 September 2013 (Trieste, Italy).
- Dissemination of scientific results using media for the general public (TV Capodistria <http://www.youtube.com/watch?v=l7OdLXKGBNw&feature=youtu.be>).

## 9 FUNDS NEEDED

**9.1 For basic research (investigation of biological mechanisms): 30.000 €**

**9.2 For applied research (solutions for real-world problems): 30.000 €**

**9.2 For pilot & demonstrator activities (to develop a prototype): 90.000 €**

## 10 CONCLUSION

Our method opens new frontiers in clinical and pre-clinical investigations of bilirubin physiology. We reveal that free bilirubin concentrations of healthy adults are around 10 nM. It remains to be elucidated what are the concentrations in the patients with some chronic diseases, and also if pharmacological treatments or healthy life style modifications can affect those values. All in all, our service can be useful to solve some unmet medical of wellness needs, which correlate with the antioxidant status of blood.

## **ACKNOWLEDGEMENT**

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## **REFERENCES**

[1] Mitja Martelanc, Lovro Žiberna, Sabina Passamonti and Mladen Franko, Direct determination of free bilirubin in serum at sub-nanomolar levels. *Analytica Chimica Acta*, 2014, vol. 809, 174-182.