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Highly sensitive method for quantitative determination of bilirubin in biological fluids and tissues☆

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ABSTRACT

Unconjugated bilirubin (UCB) exhibits potent antioxidant and cytoprotective properties, but causes apoptosis and cytotoxicity at pathologically elevated concentrations. Accurate measurement of UCB concentrations in cells, fluids and tissues is needed to evaluate its role in redox regulation, prevention of atherosclerotic and malignant diseases, and bilirubin encephalopathy. In the present study, we developed and validated a highly sensitive method for tissue UCB determinations. UCB was extracted from rat organs with chloroform/methanol/hexane at pH 6.2 and then partitioned into a minute volume of alkaline buffer that was subjected to HPLC using an octyl reverse phase (RP) column. Addition of mesobilirubin as an internal standard corrected for losses of UCB during extraction. Recoveries averaged 75 ± 5%. The detection limit was 10 pmol UCB/g wet tissue. Variance was ±2.5%. When used to measure UCB concentrations in tissues of jaundiced Gunn rats, this procedure yielded UCB levels directly comparable to published methods, and accurately determined very low tissue bilirubin concentrations (≤40 pmol UCB/g tissue) in non-jaundiced rats.

1. Introduction

Unconjugated bilirubin (UCB, Fig. 1) is produced from heme by the sequential action of intracellular heme oxygenase and biliverdin reductase, with both contributing importantly to the stress response. UCB serves as an antioxidant and cytoprotectant at physiological and mildly elevated concentrations [1,2], but may be neurotoxic when higher concentrations result from impairment of its uptake and/or conjugation, and often complicated by overproduction (e.g. hemolysis). Both its protective effects against oxidative stress and its toxicity for neurons (astrocytes and other cells) are related to the concentration of unbound UCB (Bf) in the plasma *in vivo*, or in the tissue culture medium *in vitro* [3,4]. Due to the ready diffusion of UCB across cell membranes [5], plasma Bf is an important regulator of intracellular UCB concentrations, which is the ultimate determinant of Bf's cytotoxicity. Intracellular levels of UCB are, however, modulated by its oxidation, its conjugation, and its export from the cells by membrane ABC transporters [6], creating uncertainties in the prediction of intracellular UCB levels from plasma Bf measurements. Thus, the ability to measure very low UCB concentrations in tissues and biological fluids (e.g. cerebrospinal fluid, [CSF]) should improve our understanding of UCB-induced cytotoxicity as well as its protective effects, even though the biological activity of intracellular bilirubin is modulated by binding to cytosolic proteins and by multiple, often inducible mechanisms that protect the cells against the oxidant, apoptotic, and other inimical effects of excessive pigment accumulation.

Determination of bilirubin in tissues and biological fluids is complicated by its sensitivity to light and oxygen, rapid degradation in both acidic and alkaline solutions, and high-affinity for proteins [7,8], as well as very low concentrations under normal

Abbreviations: Bf, free bilirubin; BHT, butylated hydroxytoluene; CSF, cerebrospinal fluid; DMSO, dimethylsulfoxide; IS, internal standard; MBR, mesobilirubin; R.S.D., relative standard deviation; TBA, tetrabutyl-ammonium hydroxide; UCB, unconjugated bilirubin.

☆ The experimental work was carried out at the Hepatology Lab, Institute of Clinical Biochemistry and Laboratory Diagnostics, 1st Faculty of Medicine, Charles University in Prague, Czech Republic.

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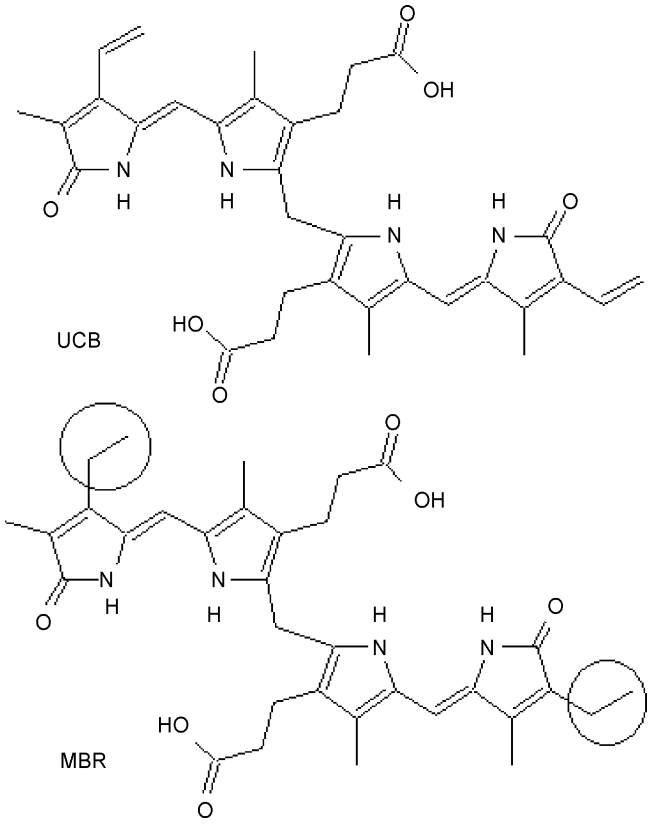

Fig. 1. Chemical structure of unconjugated bilirubin (UCB) and an internal standard mesobilirubin (MBR). Molecules differ in two double bonds (circles).

conditions. To circumvent the last complication, many studies have investigated the high tissue UCB levels in the Gunn rats (which has a severe hereditary unconjugated hyperbilirubinemia due to congenital absence of UGT1A1 [9], the enzyme that converts UCB to glucuronosyl conjugates). This mutant rat is also considered to be the best animal model to study neonatal and congenital hyperbilirubinemias in humans.

Although a number of sensitive HPLC methods for bilirubin quantification in simple matrices like serum/plasma [10], bile [11], or microsomal preparations [12] have been established, different approaches are needed for the determination of bilirubin in complex tissues. Radioassay was used to assess brain and CSF bilirubin levels after intravenous administration of [^{14}C]-UCB to Gunn rats [13,14] and guinea pigs [13]. Another approach was the determination of bilirubin and its oxidation products by ELISA using an anti-bilirubin antibody [15], which was used for bilirubin quantification in CSF of Alzheimer's disease patients [16] and in the intestinal mucosa from rats challenged with endotoxin [17]. The same antibody was used for immunohistochemical determination of bilirubin in foam cells from rabbit atherosclerotic lesions [18]. Unfortunately, these methods are not generally accessible due to the commercial unavailability of radiolabeled bilirubin or anti-bilirubin antibody, and, more importantly, underestimate tissue UCB levels due to incomplete extraction of the pigment from the tissues.

The methods most often used involve the extraction of UCB from tissues with chloroform/methanol at neutral or acidic pH, followed by quantification by direct spectrophotometry or the diazo assay. Such methods were first employed and validated to assess changes in bilirubin levels in the brains of Gunn rats after birth [19] and later to compare brain UCB levels in Gunn rat pups and their analbuminemic counterparts [20]. More recently, this approach was utilized to determine UCB concentrations in the whole brain [21] and in various brain regions [22] of Gunn rat pups and their heterozygous littermates. However, the sensitivity and specificity of this method are sufficient only for determination of high UCB levels present in jaundiced tissues. Moreover, the recovery of UCB suffers from incomplete extraction at neutral pH and degradation in acidic solutions.

The present work describes a novel HPLC-based method for the determination of UCB and total bilirubin in tissues and biological fluids. Precision is afforded by simple and rapid sample preparation, followed by extraction of UCB, with correction for incomplete recovery by use of mesobilirubin (MBR, Fig. 1) as an internal standard (IS). Accurate determination of UCB levels in selected tissues from normobilirubinemic as well as jaundiced Gunn rats demonstrates the high sensitivity of this method.

2. Experimental

2.1. Chemicals

L-Ascorbic acid, 2,6-di-*tert*-butyl-4-methylphenol (BHT), UCB, bovine serum albumin (BSA) 98%, EDTA, chloroform (HPLC grade) and tetrabutyl-ammonium hydroxide (TBA, 40% in water) were purchased from Sigma (St. Louis, MO, USA). Dimethylsulfoxide (DMSO) was from Applichem (Darmstadt, Germany), heparin from Leciva (Prague, Czech Republic), *n*-hexane (Uvasol) was from Merck (Darmstadt, Germany), and MBR, hemin and biliverdin from Frontier Scientific (Logan, UT, USA). All other chemicals were of analytical grade purchased from Penta (Prague, Czech Republic).

2.2. Animals

Hyperbilirubinemic adult male Gunn rats (RHA/jj) with congenital deficiency of bilirubin UDP-glucuronosyltransferase, and their normobilirubinemic heterozygous (RHA/Jj) adult male littermates (each $n = 3$, weight range 250–270 g) were used in the study and bred at the 1st Faculty of Medicine, Charles University in Prague. The study met the accepted criteria for the humane care and experimental use of laboratory animals. All protocols were approved by the Animal Research Committee of the 1st Faculty of Medicine, Charles University in Prague.

2.3. Tissue preparation

Under intramuscular anesthesia (ketamine and xylazin, Spofa, Czech Republic), the animals were exsanguinated via the inferior vena cava. Blood was then flushed from the circulation through cannulation of the same vein with 10 mL of washing solution [NaCl 0.9% (w/v), containing 500 IU/mL of heparin, ascorbic acid (1 mg/mL), and EDTA (1 mg/mL)], until the perfusate was completely free of blood (at room temperature). Livers were excised rapidly and flushed with additional 10 mL of the washing solution via the portal vein. Other organs including brain, spleen, kidney, testis, heart, and visceral fat were also harvested. Tissues were then rinsed 2 times in the washing solution and samples were wrapped in aluminum foil, snap frozen in liquid nitrogen and stored at −80 °C until analysis.

2.4. Preparation and storage of standards

All steps were performed under dim light in aluminum-wrapped glass tubes. Bilirubin was purified and recrystallized according to McDonagh and Assisi [23]. Its molar extinction coefficient in chloroform ($E_{453\,nm}$ = 56200 L/mol cm) was comparable to the published value [24]. In addition, an HPLC analysis revealed one major peak corresponding to naturally occurring isomer bilirubin IXα (92%)