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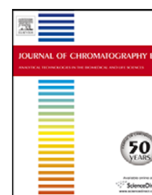

# Highly sensitive method for quantitative determination of bilirubin in biological fluids and tissues☆

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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.

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## 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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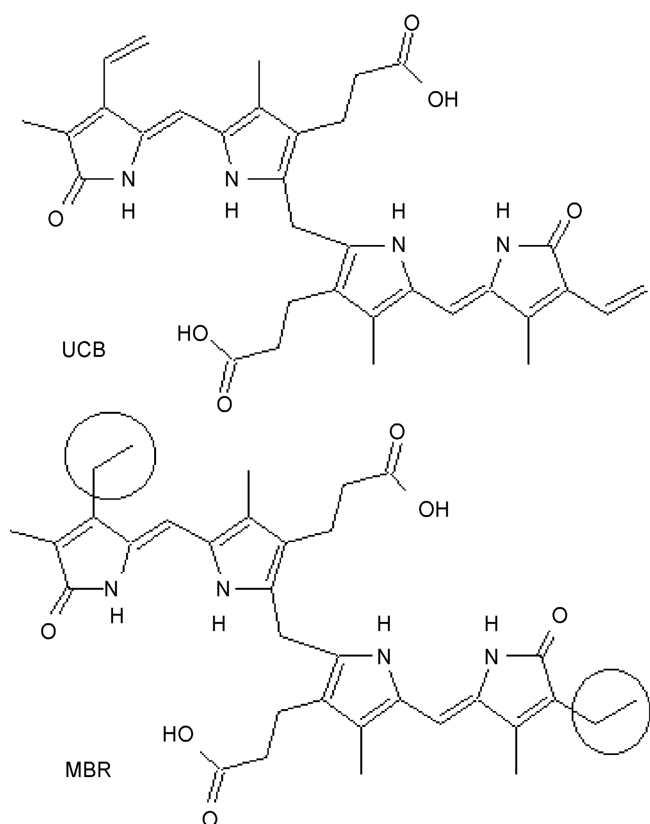


**Fig. 1.** Chemical structure of unconjugated bilirubin (UCB) and an internal standard mesobilirubin (MBR). Molecules differ in two double bonds (circles).

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%)

and two smaller peaks of isomers bilirubin IIIα (4%) and bilirubin XIIIα (4%) which are generally present in commercial UCB lots. Presence of other contaminating pigments has been excluded. Additional analyses revealed only traces (<0.1%, w/w) of other likely contaminants (bile acids [25] and cholesterol [26] by GC/MS and phospholipids by TLC [27]).

Purified UCB (10 mg) was dissolved in 12 mL of 0.1 M NaOH and immediately neutralized with 6 mL of 0.1 M phosphoric acid. The mixture was subsequently diluted with BSA solution to reach a final concentration of 480 μM UCB and 500 μM BSA in phosphate buffer (25 mM, pH 7), and serially diluted with an albumin solution (500 μM BSA in 25 mM phosphate buffer, pH 7) to yield solutions with UCB concentrations in the range 0.1–48 μM. These standard solutions were then aliquoted and stored at −80 °C for up to 6 months.

MBR dissolved in DMSO (15 μM) quickly divided into 30-μL aliquots and then stored at −80 °C, served as an IS. Due to its rapid degradation under rewarming (see Section 3), each aliquot was used only once immediately after thawing.

### 2.5. Tissue extraction

All steps were performed under dim light in aluminum-wrapped, 15-mL glass centrifuge tubes with Teflon-lined screw caps (Gerresheimer, Vineland, NJ, USA) using solvents that had been degassed by sonication. The whole procedure was performed within 30 min to minimize UCB degradation. Samples of tissue (20–200 mg) were placed in tubes containing 1 mg BHT, glass dust, and 500-μL deionized water, and 20-μL freshly thawed IS in DMSO were added. Tissue was then disintegrated to a fine suspension by vigorous grinding with a glass rod for 5 min.

The mixture was subsequently diluted with 6 mL of extraction solvent [methanol/chloroform/*n*-hexane 63:31:6 (v/v/v)] and vortexed for 30 s. Then, 4.5 mL of extraction buffer (50 mM phosphate buffer, pH 6.2; 150 mM NaCl; and 5 mM EDTA) was added, the tube was vortexed for another 30 s and then centrifuged at 1000 × *g* for 5 min. The whole lower organic phase (2 mL) containing UCB was transferred with a glass pipette into a new tube and mixed with 1.5 mL of *n*-hexane and 50 μL of loading buffer (100 mM carbonate buffer, pH 10 and 5 mM EDTA). The suspension was vortexed for 30 s and then centrifuged at 1000 × *g* for 2 min. The aqueous droplet on the surface of organic phase was aspirated into a 100-μL Hamilton syringe (Hamilton, Bonaduz, Switzerland), and immediately loaded into a 50-μL loop on the HPLC column.

### 2.6. HPLC analysis

Analyses were performed on Agilent 1100 HPLC instrument with a diode array detector (Agilent, Santa Clara, CA, USA). The pigments were separated on an octyl reverse phase (RP) column with safety precolumn (Luna C8, size 4.6 mm × 150 mm, particles 3 μm/100 A, Phenomenex, Torrance, CA, USA) using methanol/water/TBA, 59:40:1 (w/w/w) as an isocratic mobile phase at 0.5 mL/min (pH adjusted to 9.0 with phosphoric acid). Column temperature was kept at 40 °C. The absorbance of the eluted pigments was monitored at 440 nm with 550 nm as a reference wavelength. A calibration curve was constructed from 100-μL aliquots of standard UCB solutions (prepared as described above). Five points in the range 0.01–4.8 nmol per sample were measured in duplicates.

In addition, octadecyl (Nucleosil C18, size 4.0 × 125 mm, particles 5 μm/100 A, Macherey-Nagel, Duren, Germany) and phenylhexyl (Gemini C6-Phenyl, size 4.6 × 150 mm, particles 3 μm/110 A, Phenomenex, Torrance, CA, USA) columns were used for screening of sorbent capability to resolve UCB and MBR.

### 2.7. Determination of recovery

Influence of tissue type and the UCB concentration on extraction recovery was determined. Purified UCB was dissolved in DMSO to concentrations 500 nM or 500 μM, aliquoted in 10-μL aliquots and stored frozen at −80 °C. Aliquots were either directly analyzed with HPLC or admixed to various biological matrices and processed as described above. In the former case, aliquots were mixed with IS, diluted to 100 μL with loading buffer and directly loaded onto HPLC column. In the latter case, aliquots and IS were admixed to samples of serum, liver, or brain tissue with known UCB concentration and processed as described above. Responses of directly loaded and processed UCB and MBR were compared to determine extraction efficiency and influence of tissue type and concentration of pigments.

Recovery of UCB by extraction was confirmed using radiolabeled bilirubin [$^3$H]UCB, prepared according to Webster et al. [28] and Bayon et al. [29] from dog bile using tritium labeled 5-aminolevulinic acid (GE Healthcare, Uppsala, Sweden) as a biosynthetic precursor. [$^3$H]UCB was purified by recrystallization as UCB standards (>99% spectroscopic purity), samples (100 mg) of normobilirubinemic rat livers were homogenized as above and 10 nmol of purified [$^3$H]UCB (specific activity = 0.95 Ci/mol) were added. After 5 min of incubation, the suspension was processed as described above. Samples from each extraction step were taken, diluted 1:100 to 1:1000 with scintillation liquid (Rotiszint 22, Carl Roth, Karlsruhe, Germany), equilibrated for 16 h and measured in scintillation counter (RackBeta 1219, LKB-Wallac, Turku, Finland) with internal correction.

### 2.8. Method validation

A sample of liver tissue from one jaundiced animal was processed 10 times within 1 day and 10 times during 10 successive days and a relative standard deviation (R.S.D.) of results was calculated to determine within-day and between-day precision of the method, respectively. Samples and standards in different phases of the procedure were stored at room temperature or frozen for relevant time period and their responses at HPLC were compared to determine stability of UCB and MBR and method robustness. Limit of detection was calculated as a concentration of UCB at which HPLC signal to noise ratio was 3 when 200 mg of tissue was assayed. Specificity of the method was determined using interference assay with hemin and biliverdin (in concentrations 100 nmol/g tissue) as potential interfering substances. Hemin was dissolved as described for UCB standards, and biliverdin was dissolved in distilled water.

### 2.9. Determination of conjugated bilirubin

To determine amount of conjugated bilirubin in the liver tissues of RHA/Jj animals, a hydrolysis step was added to the extraction procedure. Samples of liver tissues (100 mg) with an IS, 1 mg of BHT, and 0.5 mg of ascorbic acid in the tube with 0.5 mL of water were homogenized using glass dust. Then 50 μL of 1 M NaOH was added, suspension was vortexed and incubated in the dark at room temperature to allow hydrolysis of bilirubin conjugates. After 10 min, the samples were neutralized with 250 μL of 0.1 M $H_3PO_4$ and UCB was extracted as described above.

### 2.10. Comparison with previously published methods

Serum, brain, and liver bilirubin content were determined using either our new method or chloroform/methanol extraction followed by direct spectrophotometry [19] or the diazo assay [20]. Hyperbilirubinemic sera and tissues from Gunn rats (RHA/jj, *n* = 3)

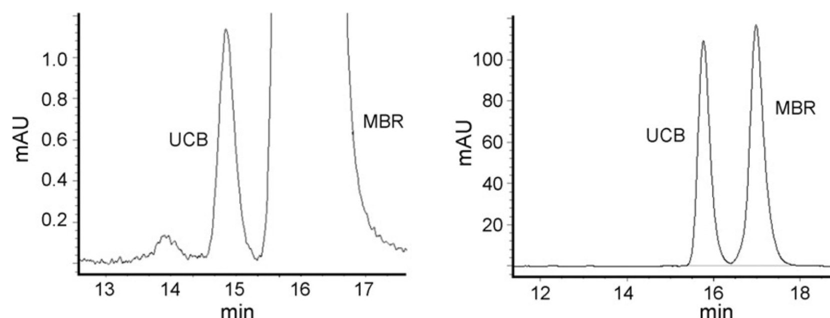


**Fig. 2.** (a) A typical chromatogram of a sample from the spleen of a normobilirubinemic rat. (b) A typical chromatogram of a sample from the visceral fat of a normobilirubinemic rat. Analyzed pigments form separated peaks without any interference from samples even at UCB levels close to the detection limit of the method. UCB: unconjugated bilirubin; MBR: mesobilirubin. No other bile pigment peaks were seen (e.g. biliverdin, oxidative derivatives of bilirubin).

were studied, due to the inadequate sensitivity of the other methods with tissues from non-jaundiced animals.

## 3. Results and discussion

### 3.1. Tissue extraction

All steps of the method were optimized in preliminary studies. Homogenization properties of ultrasound and Dounce homogenizers were found to be just as effective as glass dust, which enabled maximal recovery of sample and minimal time delay. Saturation of solvents with argon and use of antioxidants other than ascorbate and BHT had no additional protective effect on bilirubin degradation. The optimal pH range for pigment extraction from tissues was found to be 6.0–6.5. Use of higher pH resulted in incomplete extraction while lower values led to UCB degradation, probably related to co-extraction of prooxidant hemin. In the first extraction step at pH 6.2, proteins, salts and other polar substances were cleared from the organic phase, in which the UCB diacid was retained. In the second extraction at pH 10, lipids remained in the organic phase and UCB and MBR dianions were extracted to a polar phase optimal for loading onto the HPLC column.

Repeated determinations of UCB in a representative liver tissue sample showed a very good within-day ($n$ = 10, R.S.D. = 2.5%) as well as between-day precision ($n$ = 10, R.S.D. = 2.7%). Extraction recoveries were 75 ± 5% with 3% of pigment lost in the aqueous phase and 2% in the protein interphase during the first extraction at acidic pH, while 15–25% was lost during organic phase transfer and reversed extraction into aqueous droplet at alkaline pH. Recovery was independent of UCB concentrations and virtually identical for UCB mixed with sera or various tissues. Tissue UCB, UCB stabilized with albumin and MBR dissolved in DMSO were stable when stored frozen at −80 °C for at least 6 months. UCB and MBR in the organic solvent phase after the first extraction were stable for at least 1 h at room temperature or 18 h at −20 °C. However, MBR in DMSO was unstable at room temperature (10% degradation within 1 h). Both pigments were comparably unstable in the alkaline loading buffer (15% degradation within 20 min). To minimize losses, each sample was loaded onto the HPLC column within 5 min after extraction.

### 3.2. HPLC assay

Again, various parameters of HPLC analysis were optimized. MBR, a UCB derivative with a closely similar structure (Fig. 1) and physicochemical properties was found to be an optimal IS since it does not occur in biological tissues. The behavior of both pigments in samples, i.e. their stability as well as extraction efficiencies were virtually identical as their ratio did not change during the procedures. The octyl (C8) column was shown to resolve UCB and MBR sufficiently whereas neither octadecyl (C18) nor phenylhexyl (C6-Phenyl) columns separated them (data not shown). The mobile phase contained TBA as an ion pair agent to ensure optimal peak sharpness and maximal sensitivity [10].

Mobile phase strength, flow and column temperature were adjusted to reach retention times of UCB and MBR in the range of 15–20 min. Under these conditions, separation of both pigments was sufficient with peak overlap about 1% (Fig. 2). Detection wavelength 440 nm close to the absorption maxima of both substances (452 nm for UCB and 425 nm for MBR dissolved in the mobile phase) was selected to ensure maximal sensitivity. No coeluting substances were found in either tissue sample, and neither hemin nor biliverdin interfered with the procedure. The detection limit of the HPLC assay was 1.5 pmol UCB/sample and the absolute detection limit of the whole determination was 10 pmol/g tissue (equals to 10 nM concentration in fluids), which is sufficient for precise UCB determination in ≤100 mg of all the tissues investigated. Calibration curve was linear in a broad range from 0.01 to 4.8 nmol/sample ($y$ = 0.98(nmol/sample), $r^2$ = 0.9997). Depending on the recommended amount of tissue (20–200 mg per sample) and the linearity of the calibration curve, the method was capable of quantifying tissue bilirubin levels in the range 0.05–240 nmol/g tissue (corresponding to 0.05–240.00 μM UCB in fluids).

### 3.3. Conjugated bilirubin

To determine total bilirubin content present in RHA/Jj rat liver tissue bilirubin conjugates were hydrolyzed in 0.1 M NaOH before UCB extraction. Alkalization of one sample of liver tissue for 30 s to 15 min resulted in complete hydrolysis after 6 min (Fig. 3), without

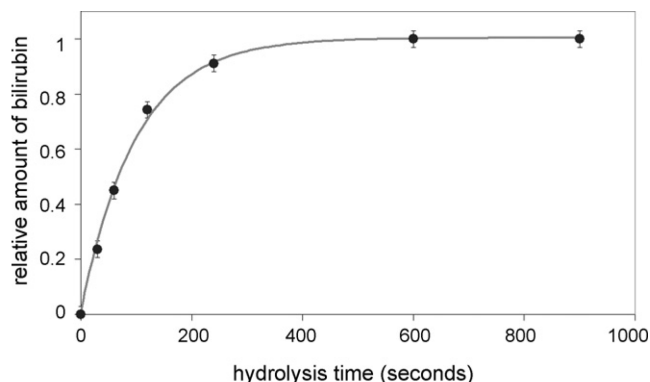


**Fig. 3.** Time course of alkaline hydrolysis of bilirubin conjugates in rat liver. Normobilirubinemic rat liver tissue (RHA/Jj) was hydrolysed in 0.1 M NaOH. Hydrolysis was measured as increment of UCB in reaction mixture determined with our novel method. Experimental points represent the mean from two measurements, sections correspond to interquartile range. Curve represents ideal first order reaction kinetics. UCB: unconjugated bilirubin.

**Table 1**
Bilirubin concentrations in various organs/tissues of hyperbilirubinemic and normobilirubinemic rats

| Tissue | RHA/jj, $n = 3$ (nmol/g) (median, 25–75% range) | RHA/Jj, $n = 3$ (nmol/g) (median, 25–75% range) | Ratio (jj/Jj) |
|---|---|---|---|
| Liver–UCB | 88 (73–91) | 0.70 (0.65–0.75) | 126 |
| Liver–TBIL | N.D. | 1.6 (1.4–1.7) | N.D. |
| Liver–CBIL | N.D. | 0.87 (0.72–0.97) | N.D. |
| Brain | 3.0 (2.7–3.2) | 0.089 (0.08–0.09) | 34 |
| Myocardium | 21 (20–24) | 0.16 (0.15–0.18) | 131 |
| Visceral fat | 12.1 (11.5–12.4) | 0.047 (0.04–0.05) | 255 |
| Kidney | 33 (31–43) | 0.23 (0.19–0.24) | 144 |
| Spleen | 16.0 (14.9–16.1) | 2.9 (2.3–6.5) | 5.5 |
| Testis | 11.7 (11.7–14.8) | 0.32 (0.29–0.33) | 37 |
| Blood serum | 179 (164–190) | 0.46 (0.45–0.51) | 389 |

UCB: unconjugated bilirubin; TBIL: total bilirubin; CBIL: conjugated bilirubin; N.D.: not determined.

signs of UCB degradation up to 15 min. To be sure that hydrolysis was complete, incubation in NaOH was performed for 10 min when determining total bilirubin in liver samples from heterozygous rats (RHA/Jj, $n = 3$). Conjugated bilirubin concentration in livers was calculated as total bilirubin minus UCB (Table 1).

### 3.4. Comparison with other methods

UCB concentrations in Gunn rat sera, livers, and brains ($n = 3$ each) were determined, comparing our HPLC method with two previously published methods [19,20] (Fig. 4). Under these conditions, results of previous methods were comparable to our novel approach suggesting that precision is similar at high UCB levels (data not shown). However, both the diazo assay [20] and especially direct spectrophotometry [19] are known to be sensitive to interferences from diazo-positive substances and various pigments which decrease their accuracy at lower UCB levels. In contrast, our method uses highly specific and sensitive HPLC quantification and interferences are unlikely. Furthermore, bilirubin levels in livers and brains of normobilirubinemic rats were undetectable using the older methods [19,20], even when 750 mg of tissue was analyzed (data not shown).

The results are in line with published data [19–22], and demonstrated that previously published methods were limited by insufficient sensitivity due to their much higher detection threshold. Most studies reported only tissue UCB levels in brains of newborn Gunn rats or RHA/Jj rats with high bilirubin content due to immaturity of the blood/brain barrier. In these cases, brain UCB concentrations were found to range from 4.3–5.3 nmol/g in 15–19-day-old Gunn rat pups [19–22]. Tissue bilirubin concentrations in adult Gunn rats, studied only by Sawasaki [19], were found to be $1.7 \pm 0.3$ and $85 \pm 15$ nmol/g in the brain and liver, respectively. This data is in a good agreement with our method (Table 1), which, however, could also determine bilirubin concentrations in organs of normobilirubinemic rats.

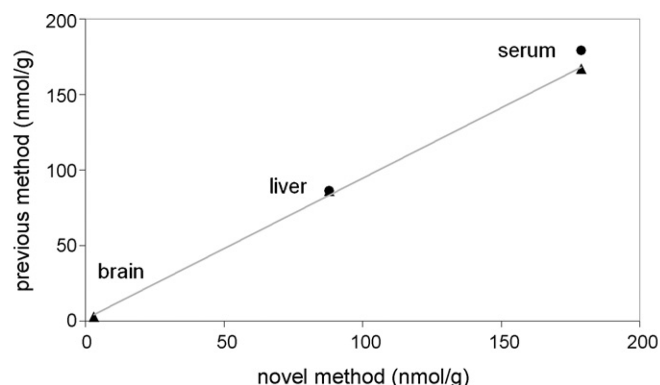


**Fig. 4.** Comparison of results from our new and previously published methods. Tissue UCB levels in brain, liver and blood serum of jaundiced (jj) Gunn rats ($n = 3$ each) are strongly correlated between the old and our new methods ($r = 0.9993$), suggesting similar accuracy at high bilirubin levels. Old methods: (●) direct spectrophotometry [19]; (▲) diazo assay [20]. UCB: unconjugated bilirubin.

### 3.5. UCB levels in various tissues of jaundiced and non-jaundiced rats

UCB concentrations were assayed in various organs/tissues and sera of adult hyperbilirubinemic Gunn (RHA/jj) rats as well as their normobilirubinemic heterozygous (RHA/Jj) littermates (Table 1). UCB levels in organs from heterozygous animals ranged from 0.047 to 2.9 nmol/g in the visceral fat and spleen, respectively, while in jaundiced animals ranged from 3.0 to 88.0 nmol/g in the brain and liver, respectively. Not surprisingly, tissue UCB levels were substantially higher in jaundiced animals as compared to their heterozygous littermates in all investigated organs. However, the relative elevation of UCB content in the jaundiced animals differed among organs (Table 1), ranging from 5.5-fold in the spleen, and 34-fold in the brain, to 255-fold in the visceral fat and 389-fold in sera.

The variability among tissues is most likely due to differences in production, uptake, intracellular binding, conjugation, oxidation, and excretion of UCB [4]. High levels in the spleen of both rat strains are probably due to splenic function as the primary site of catabolism of red-cell heme to UCB, the rate of which is unaltered in the jj rats [30]. High bilirubin levels in the liver are likely to reflect the facilitated diffusion for uptake, and the high concentrations of ligandin, a high-affinity cytosolic binding protein for UCB, in this organ. The high concentrations of serum albumin and its high-affinity for UCB account for the high UCB levels in blood serum [8]. The relatively low levels in the brain in both jaundiced and non-jaundiced rats may be attributed to the absence of ligandin and the presence of ABC export pumps for UCB (Mrp1 in the choroid epithelium and Mdr1 in the brain capillary endothelial cells (blood–brain barrier) [4]. The relatively low increment in brain UCB in the jaundiced rats could be explained by the induction and translocation of Mrp1 in brain cells exposed to high levels of UCB, as demonstrated *in vitro* for astrocytes [31]. The marked increment in UCB levels in the visceral fat of jaundiced rats is known for decades but remains somewhat surprising, since UCB is not highly soluble in triglycerides [7]. Whatever the mechanism, the high levels of UCB in the large mass of visceral fat provides a major alternative storage site to protect the brain from toxic accumulation of retained UCB; one might speculate that the greater susceptibility of premature infants [32] and Gunn rat pups [33] to develop bilirubin encephalopathy at lower plasma UCB concentrations may be related in part to their relative paucity of visceral fat.

## 4. Conclusions

A highly sensitive and precise method for quantification of UCB in biological fluids and tissues is presented. Rapid and sim-

ple sample preparation enables good recovery, even from complex biological matrices. The final HPLC assay is not only highly sensitive and specific, but the use of MBR as an IS corrects for losses during extraction. Addition of a hydrolytic step also allows for the determination of conjugated bilirubin. Determination of tissue UCB levels with our new method was possible in all the investigated organs and were comparable to those obtained with several previously published methods when applied to tissues from jaundiced Gunn rats. Unlike the older methods, its enhanced sensitivity permitted accurate determination of tissue bilirubin levels even in normobilirubinemic animals, using as little as 100 mg of tissue. Thus, our method is rapid, versatile, precise and sufficiently sensitive for assessment of bilirubin concentrations in cells, tissues and biological fluids, even in the physiological range.

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