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Bilirubin measurement – an updated survey[1)]

Markus Thaler[1], Peter B. Luppa[1,*] and Harald Schlebusch[2]

[1] Institut für Klinische Chemie und Pathobiochemie, Klinikum rechts der Isar, Technische Universität München, München, Germany
[2] Nadistraße 14, 80809 München, Germany

Abstract

The goal of precise, accurate and specific measurement of bilirubin and its subfractions in serum has not yet been achieved, despite many different methods being available. In addition to the traditional photometric diazo procedures, drychemistry methods, direct spectrophotometry in serum and whole blood and for scientific purposes separations by HPLC were established. For point-of-care diagnostics several test procedures are available. This article reviews the most notable methods and describes advantages and disadvantages of the different bilirubin analyses.

Keywords: bilirubin; methods; point-of-care diagnostics.

Bilirubin isomers and fractions

Bilirubin in plasma is not a single homogeneous substance but represents a variable mixture of isomers and fractions. The various "bilirubins" differ, sometimes considerably, in their physiological behaviour and their chemical reactions in vitro.

Unconjugated bilirubin (Bu)

In the most part *Bu* derives from the breakdown of haemoglobin (Hb) of aged erythrocytes. The quantitatively predominant form arises through the splitting of the haem ring in the IX-α position and hence is designated (Z,Z)-bilirubin IX-α. However, there are other isomers that are generated through splitting of the haem ring in the β, γ or δ position. Bilirubin IX-α is virtually insoluble in water at physiological pH, since its carboxy and amino groups are fixed by intramolecular hydrogen bonds (Figure 1A). These intramolecular hydrogen bonds also determine the basin-shaped topology of *Bu* (Figure 1B). The β, γ or δ isomers cannot form any hydrogen bonds, and are therefore more readily water-soluble and can be excreted through the kidneys. There are other isomers such as bilirubin III-α and bilirubin XIII-α that, although they do not occur in the plasma, can nevertheless be present in large quantities in standard preparations.

Conjugated (glucuronated) bilirubin (Bc)

The transformation of albumin-bound bilirubin to its water-soluble gucuronide takes place in two steps. Both the initial monoglucuronide and also the diglucuronide that forms in a subsequent step can occur as C8 or C12 isomer. In the plasma of healthy adults and generally also in icteric neonates, there are only very low concentrations of conjugated bilirubin (<0.1 mg/dL). The "direct" bilirubin that can be detected in these sera (up to 1.5 mg/dL in neonates, depending on the method) is not conjugated but unconjugated bilirubin (see chapter "direct" bilirubin).

Delta-bilirubin (Bd)

Delta-bilirubin arises through a non-enzymatic covalent coupling reaction between glucuronated bilirubin and albumin, so this fraction is found in patients with hepatic and posthepatic icterus or with Dubin-Johnson syndrome. It is, on the other hand, not found in prehepatic icterus or in neonates with unconjugated hyperbilirubinaemia. Delta-bilirubin is not excreted by liver or kidneys, but is slowly metabolized with a half-life of ~20 days. It can constitute up to 90% of the total bilirubin in the convalescence phase of cholestatic disorders.

Free bilirubin (Bf)

Free bilirubin, i.e., unconjugated and not bound to albumin, represents a significant component of the neurotoxicity of bilirubin, which is made responsible for the bilirubin encephalopathy of the neonate (resulting in kernicterus). The toxic effect is thought to occur even at a concentration of ~0.005 mg/dL – the solubility limit of free bilirubin at physiological pH. Although from a clinical point of view there are some arguments for the impor-

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*Correspondence: Prof. Dr. Peter B. Luppa, Institut für Klinische Chemie und Pathobiochemie, Klinikum rechts der Isar der Technischen Universität München, Ismaninger Str. 22, 81675 München, Germany
Tel.: +49 (089) 4140-4759
Fax: +49 (089) 4140-4875
E-mail: luppa@klinchem.med.tum.de

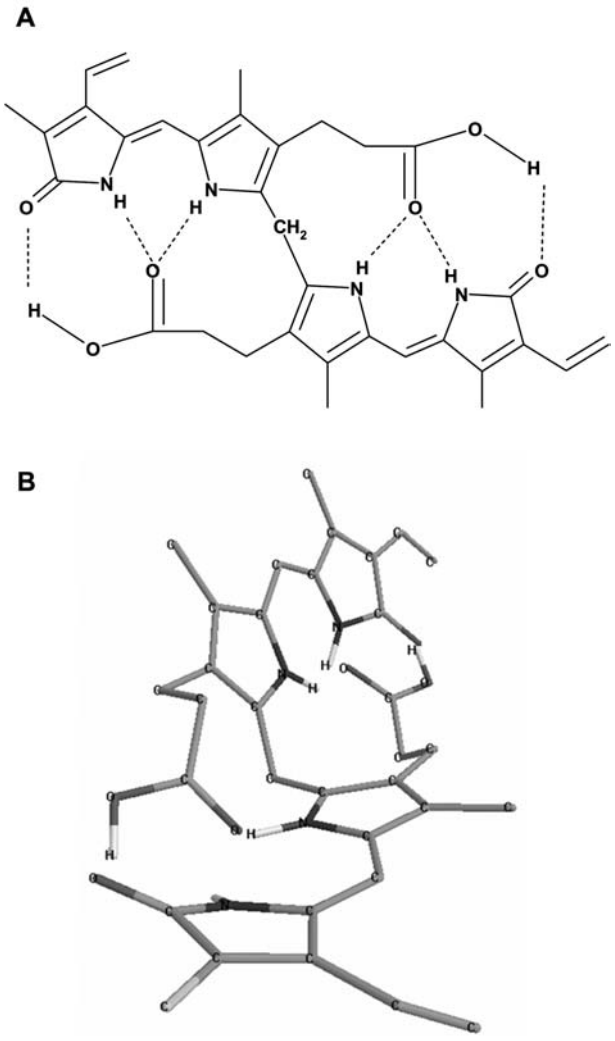

Figure 1 Structural formula (A) and topology (B) of unconjugated bilirubin.

tance of free bilirubin, from the analytical point of view this fraction remains hypothetical. Up to now, there has been no really reliable method for measuring free bilirubin in plasma (or alternatively for measuring the free binding capacity of albumin for free bilirubin).

Bilirubin fractions in phototherapy

Phototherapy, which uses visible light at wavelengths of 420–470 nm, is today the standard therapy for hyperbilirubinaemia of medium severity in neonates. Together with photooxidation products, i.e., fragments of the molecule, two new fractions are produced through a cis-trans isomerization (Z→E): the stereoisomer (4Z,15E)-bilirubin IX-α and to a lesser extent (4E,15Z)-cyclobilirubin IX-α. Through the "switch" of a double bond, one of the outer rings of the molecule is so rotated that the formation of a hydrogen bond is no longer possible. The molecules are therefore more polar and better water-soluble, and are mainly excreted without conjugation by the liver and bile. The disruption of the molecule through photooxidation plays quantitatively only a minor role. The therapeutically pivotal process is the isomerization. Immediately after completion of phototherapy, (4Z,15E)-bilirubin IX-α can constitute up to 20% of the total bilirubin, and (4E,15Z)-cyclobilirubin IX-α can be up to 3%. (4Z,15E)-bilirubin IX-α is eliminated with a half life of about eight hours, and cyclobilirubin with a half life of about three hours. In contrast to cyclobilirubin, the quantitatively predominant fraction is "diazo-positive", so that most routine methods cannot distinguish between the potentially toxic unconjugated (4Z,15Z)-bilirubin IX-α and the nontoxic (4Z,15E)-bilirubin IX-α [1–4].

Measurement of total bilirubin

Reference method (CLSI)

In 1985, Doumas et al. described a "Candidate Reference Method" for the measurement of total bilirubin in serum [5], and this has subsequently been recognized as the reference method by the Clinical and Laboratory Standards Institute (CLSI), formerly the National Committee for Clinical Laboratory Standards (NCCLS) [6]. The basis of this method is the Jendrassik-Grof procedure, which is optimized with respect to the concentration of the reagents, the reaction time and a range of marginal conditions [7].

For testing sample and blank, 0.5 mL of serum is mixed with 4.0 mL of caffeine reagent. After ten minutes, 1.0 mL diazoreagent (sample) or 1.0 mL sulfanilic acid (blank) is added, and after further ten minutes 3.0 mL of tartrate solution is added. After ten minutes, photometry is performed at 598 nm.

The method is calibrated with the Standard Reference Material SRM 916a of the National Institute of Standards and Technology (NIST), which contains unconjugated bilirubin at a purity of >99%. The material must be dissolved (by means of dimethyl sulfoxide (DMSO) and Na_2CO_3) in human serum or in a 40 g/L solution of albumin. Other highly pure bilirubin preparations (e.g., Pfanstiehl bilirubin) are also suitable standard substances [8]. However, it has been shown that, for calculation of the results, the molar extinction coefficient of azobilirubin (76490 L/mol/cm) can also be used [9]. For practical purposes the volumes used for the reference method can be reduced without worsening of precision and accuracy [10]. The method is linear to at least 25 mg/dL.

It is not clear whether *Bd* is really 100% converted [5, 11]. The interference through haemoglobin that is typical for the diazo methods is only apparent in the sample at Hb concentrations of >2 g/L. In this case, the addition of ascorbic acid (0.1 mL per 40 g/L solution) before addition of the tartrate reagent is recommended. A general addition of ascorbic acid to every sample is, however, not recommended, since ascorbic acid destroys the diazo reagent [12].

Routine methods based on coupling with diazonium salts

The classical methods of bilirubin measurement are based on the azo-coupling of bilirubin and diazonium ions at the central methylene group of bilirubin, first described by Ehrlich in 1883 [13]. Bilirubin reacts with diazotized sulfanilic acid under formation of an azopigment and hydroxypyrromethene-carbinol. The latter reacts with a further molecule of diazotized sulfanilic acid, resulting in the splitting off of formaldehyde and the formation of a second molecule of azopigment [14]. As a result, this reaction produces from unconjugated bilirubin two diazotized isomers and from bilirubin-monoglucoronide and -diglucoronide two different glucuronidized azopigments and two non-esterified monomers each [15]. The azopigments produced serve as indicators [13]. In general *Bc* and *Bd* react rapidly with diazonium salts but *Bu* only slowly. The reason for this are the above-mentioned hydrogen bonds of *Bu* (Figure 1A), which make the central methylene group sterically inaccessible. For measuring of total bilirubin it is therefore necessary to add so-called accelerators, such as, for example, caffeine, methanol, ethanol, sodium acetate, sodium benzoate, or diphyllin. These substances weaken the intramolecular hydrogen bonds of *Bu* and allow its release from albumin, so that *Bu* can then also react rapidly [13, 15, 16]. Many modifications of the original diazo-method have been proposed, of which the most important will be described briefly below.

The *Malloy-Evelyn* method uses methanol as accelerator, leading to improved solubility of the resultant azobilirubin [17]. However, methanol can also precipitate proteins, and this possibly could lead to falsely low values for total bilirubin.

Jendrassik and Grof have described an improved procedure [7], characterized by higher precision and reliability with decreased interference; currently, this forms the basis of most of the automated systems for bilirubin testing that employ the azoreaction. Caffeine and sodium benzoate are used as accelerators, and diazotized sulfanilic acid is, as usual, used as diazo component. After the completion of an appropriate reaction time, the addition of ascorbic acid, which destroys the excess diazopigment, finally blocks the azo-coupling. In a neutral medium the reaction products shows a red colour at an absorption maximum of 530 nm. The addition of alkaline tartrate leads to a shift in the absorption maximum to a wavelength of 598 nm, at which the solution takes on a blue colour. The bilirubin content can then be determined by photometric quantification of the colour change [13, 16].

A further procedure frequently used in routine clinical diagnostics is the *DPD method*: in an acid medium with 2,5-dichlorophenyl-diazonium-tetrafluoroborate (DPD), bilirubin forms an azopigment that can be measured photometrically at 540 to 560 nm. The release of the *Bu* from the albumin is achieved with the detergent Triton X-100, which also helps to avoid protein precipitations [16, 18]. There is good agreement between the measurement results of the Jendrassik-Grof principle and the DPD-method. However, the latter is much less laborious.

The *DCA* method is also worth mentioning; it uses 2,4-dichloroaniline (DCA) as diazonium compound, originally with methanol as accelerator [19]. Alternatives are ethylene glycol [20] or the detergent Brij35 [21]. An advantage compared to the procedures that are based on diazotized sulfanilic acid is that 2,4-dichloroaniline can be stored longer. A disadvantage, however, is the high susceptibility to haemolysis.

Enzymatic measurement

Isolation and purification of bilirubin oxidase (BOX, EC 1.3.3.5) from the fungus species *Myrothecium verrucaria MT-1* has permitted the development of an enzymatic method for measuring total bilirubin. BOX is a 52 kDa enzyme with one copper ion attached to every enzyme molecule, and with a maximum activity at pH 8.0. BOX is completely inhibited by Fe^{2+} and KCN, and partially inhibited by sodium azide, thiourea and NaCl [22, 23]. It catalyses the oxidation of bilirubin to biliverdin in the presence of molecular oxygen. At pH values of 5.0–8.5, the biliverdin is further oxidized to purple products that finally become colourless, whereas at a pH of <4.7 the biliverdin persists in the reaction mixture for several days [14]. Total bilirubin is measured with the enzymatic method at a pH of 8.2 after the addition of sodium dodecyl sulfate and sodium cholate; the two additives are required to release the bilirubin that is bound to albumin, which then, just like the unbound bilirubin, can be rapidly converted by BOX. Therefore under the above-mentioned conditions all bilirubin fractions of the serum (including the conjugated fractions) are oxidized to biliverdin. The total bilirubin can be quantitatively determined either on the basis of the decrease in the absorption of bilirubin at 425 nm or through the increase in the absorption of the purple pigment at 450 nm. An advantage of the enzymatic method is the low amount of required sample. However, the values obtained for total bilirubin are somewhat lower than those obtained by the diazo-procedure [14–16, 24].

Spectrophotometric methods following dilution

In 1972 Hertz, Dybkaer and Lauritzen studied for the first time systematically the absorption spectra of unconjugated and conjugated bilirubin and developed a measurement procedure in which the serum sample is diluted with borate buffer and the reaction mixture is measured at 466 and 522 nm [25]. Vink [26] achieved an important improvement through the replacement of the borate buffer by caffeine benzoate solution. With the use of a pho-

tometer with monochromatic light (spectral band width $\leq$2 nm) the method does not need to be calibrated.

The bilirubin concentration is calculated from the following formula:

c (μmol/l) = f × 21.26 × (E 465 nm – E 528 nm)

where f = volume of the test/volume of the sample.

Interferences through oxyhaemaglobin and turbidity are very well corrected and the agreement with the CLSI reference method is excellent [27]. However, since the method cannot be automated, its use remains restricted to situations where sample numbers are low.

Spectrophotometric measurement without dilution ("bilirubinometer")

A particularly simple variant of direct spectrophotometry is used in many neonatology departments for the measurement of bilirubin in neonates. The so-called bilirubinometer is usually a simple filter photometer, which measures the absorption of the plasma at 455 nm – near the absorption maximum of bilirubin. Since neonate plasma contains no lipochromes that likewise absorb around this wavelength (e.g., carotene), bilirubin can be determined quantitatively if the spectral interference through haemoglobin is compensated. This occurs through an additional measurement at 575 nm. Since the molar extinction coefficient of the haemoglobin is identical at 455 nm and 575 nm, the bilirubin concentration can be calculated from the difference

ΔE = E 455 nm – E 575 nm.

A haematocrit capillary tube serves as cuvette; it is filled with (capillary) blood and centrifuged in a special centrifuge. Finally, the capillary tube is placed in the measuring apparatus so that the measuring slit of the photometer is completely covered by the plasma column. In general, 20–30 μL of blood is sufficient for the assay.

In the past, lack of linearity and problems with calibration have been reported to be significant causes of false results with the bilirubinometer. However, with more recent kits these problems appear largely to be solved. The round-robin tests for neonatal bilirubin of the Reference Institute for Bioanalysis of the German Society for Clinical Chemistry and Laboratory Medicine (DGKL) [28] have shown in recent years that the reference method values of the control samples are easily reached and the scatter of bilirubinometer results under comparable conditions is in part even lower than those of the classical wet-chemistry methods (e.g., Jendrassik-Grof or DPD/DCA).

The "point-of-care testing" of neonate bilirubin has been further simplified through the newly developed "Bilimeter 3" (Pfaff medical). Together with technical improvements, it is particularly noteworthy that the kit is calibrated automatically when being switched on, and the zero adjustment can be re-set by keystroke. The linear range reaches up to bilirubin concentrations of 28 mg/dL. Internal quality control is performed with a testing probe of coloured glass (with target value) or with control sera. For this apparatus too, the blood sample has to be centrifuged in a haematocrit capillary tube. The obtaining of plasma may be further simplified with the help of a "separator", which uses a fleece strip and will soon be available. However, instead of 20 μL blood at least 60 μL will be needed.

Direct spectrophotometry in whole blood (blood gas analysers)

The direct, spectrophotometric measurement of total bilirubin in whole blood is possible with the kits ABL 730, 735, 830 Flex, 835 Flex and 837 Flex (Radiometer), Rapidlab 1200 (Siemens Medical Solutions) or cobas b 221 (formerly OMNI S) (Roche Diagnostics). The measurement principle is identical for all kits [29]: in the CO-oximetry module, bilirubin is determined in the haemolysed (ABL, cobas) or not-haemolysed (Rapid lab) sample together with the haemoglobin fractions by means of a multi-wavelength measurement (ABL, 128 wavelengths in the range 478–672 nm; cobas, 512 wavelengths in the range 460–660 nm, Rapidlab 256 wavelengths in the range 500–680 nm). Although the spectra of bilirubin and haemoglobin can be clearly differentiated from each other, the great disparity in concentration between bilirubin and the interfering haemoglobin places great demands on the measurement process. The bilirubin concentration is calculated from the results of the measurements of absorption with the help of a multi-component analysis. The algorithm used has been altered several times in the past.

For the measurement, 35 μL blood is necessary for the ABL kits, and 50 μL for cobas b 221. For all kits the range of measurement reaches from 3 to 30 mg/dL. A quality control is possible with control samples supplied by the respective manufacturer. The available evaluations almost exclusively refer to neonate bilirubin, i.e., samples with unconjugated bilirubin. Despite few critical voices [30], agreement with classical laboratory methods is generally reported to be good, including the range of concentrations relevant to therapy [31, 32].

Measurements with carrier-bound reagents ("dry chemistry")

Multilayer film technology (Vitros, Ortho-Clinical Diagnostics)

The Vitros kits are, with exception of the Vitros DT60 II, fully automatic analytical kits for the quantitative measurement of clinical-chemical parameters with the help of

multilayer film technology. The required reagents are applied to a thin film, that together with a carrier layer forms a "slide" with a reaction zone of about 1 cm^2. A serum or plasma sample is applied and low-molecluar components and water diffuse into the underlying layer, the latter thereby dissolving the reagents enclosed in the slide. After completion of the indicator reaction, the pigment formed is measured reflectometrically through the transparent carrier layer. On the fully-automated Vitros kit, two different methods of bilirubin measurement are employed, one of which is based on a modified diazo-procedure and the other on a variant of direct spectrophotometry.

Determination of total bilirubin by the diazo-procedure (TBIL slide) [33, 34] The slide contains as reagents a stabilized diazonium salt (4-(N-carboxy-methyl-aminosulfonyl)-benzoldiazoniumhexafluorphosphate) as well as diphyllin and Triton X-100 as accelerators. All bilirubin fractions react quantitatively to a pigment, which is bound to a mordant and measured at 540/460 nm. The range of measurement reaches from 0.1 mg/dL to 27 mg/dL. Calibration is performed with standards supplied by the manufacturer, which are based on the reference material NIST SRM 916a. Haemoglobin interferes by two mechanisms. On the one hand the absorption spectra of haemoglobin and azopigment overlap, which leads to a positive (photometric) error. Simultaneously, the known (chemical) interference with the diazo reaction occurs, leading to a negative error. At a bilirubin concentration of 6 mg/dL and a haemoglobin concentration of 1 g/L the effects cancel out each other [35].

According to the manufacturer's information the test should not be used for samples from neonates of <14 days old, for which the *BuBc* slide should be applied. This recommendation is based on the different calibration-procedures. The differences between TBIL and *BuBc* results are, however, so small [35, 36] that this recommendation appears to be unfounded, which is also consistent with the experience of the authors.

Measurement of unconjugated and conjugated bilirubin with the help of direct spectrophotometry ("BuBc slide") [37, 38] The reagent-carrying layer of the slide contains caffeine, sodium benzoate and surfactants, which cause a dissociation of bilirubin and albumin. The dissociated, unconjugated bilirubin migrates together with the bilirubin glucuronides through a barrier layer that retains proteins – and with these delta-bilirubin too. After binding to a mordant the glucuronides show an absorption maximum at 420 nm, whereas that of unconjugated bilirubin is at 460 nm. At 400 nm the molar extinction effects are virtually equal. Using a two-wavelength measurement and an elaborate calculation it is possible to determine the concentrations of unconjugated and conjugated bilirubin separately. Since haemoglobin is held back by the barrier layer, the test is practically not influenced by haemolysis [35]. The range of measurement reaches up to 27 mg/dL. Calibration is performed with standards supplied by the manufacturer, which are based on the HPLC method by Lauff [39].

If the bilirubin concentration of a sample is determined by both methods, the concentration of *Bd* can be deduced from the difference of the results. These derived values agree closely with results of HPLC measurements [40]. Further derived values are as follows: DBIL (direct b.) $= Bc + Bd$ and NBIL (neonate b.) $= Bu + Bc$.

Impregnated test-strip technique (Reflotron, Roche Diagnostics GmbH)

The "Reflotron plus" and "Reflotron sprint" are typical "point-of-care" kits that operate by the principle of an impregnated test-strip. The strip contains all reagents and additives that are necessary for the reaction. On the reverse side of the reagent carrier a magnetic strip contains information about test and lot, among others the calibration data. All measurements are performed as individual measurements from whole blood, serum or plasma.

The bilirubin test measures total bilirubin. Measurement of the direct bilirubin is not possible. The applied sample (30 μL) flows into a first reaction layer, in which indirect bilirubin is released by diphyllin. In case of using whole blood as sample material, erythrocytes are separated by a fleece layer before the rest of the sample reaches the first reaction layer. In a second stage all bilirubin fractions react with the diazonium salt 2-methoxy-4-nitrophenyldiazoniumtetrafluoroborate to an azopigment, which is measured at 567 nm in a reflection photometer. The range of measurement reaches from 0.5 mg/dL to 12 mg/dL. Because of rheological problems (including the influence of haematocrit), the system is not suitable for use in samples from neonates. For calibration, the DPD method served as comparison method. The functionality of the kit is ensured by means of automatic controls. In addition, the optical system can be checked with a control strip. For control of the entire system in the context of internal quality control, control sera are available.

Separation of the bilirubin fractions by HPLC

There are various methods for the separation and quantification of the bilirubin fractions in serum by means of HPLC. In earlier methodological studies the mono- and diglucuronide were first converted into the stable methyl ester by methanol, and then extracted together with the *Bu* with chloroform. The separation then proceeded either by normal-phase HPLC on a silica gel column or with reversed-phase HPLC on a C18 column. This procedure also permits separation of the C8 and C12 isomers of the monomethyl ester [41–43]. However, since

serum proteins are denatured and completely removed, measurement of *Bd* is not possible with this method [14].

Hence, Lauff et al. developed a HPLC method in which the serum is pretreated with a saturated sodium sulfate solution. This method precipitates mainly proteins that are larger than albumin, while albumin itself, together with *Bd*, remains in solution. The serum is then transferred to a reversed-phase column, which is eluted, according to decreasing polarity, with a linear gradient from a diminishing, acid, aqueous phase based on phosphoric acid and an increasing isopropanol-based phase. The *Bd* is eluted most rapidly through the column. Then the other bilirubin fractions follow in the order bilirubin-diglucuronide, bilirubin-monoglucuronide and *Bu* [39]. Since the precipitation with sodium sulfate is not completely selective, there is a risk that variable proportions of albumin will also precipitate leading to a loss of *Bd*. The method is also too insensitive at total bilirubin concentrations <1 mg/dL and relatively laborious [14].

By diluting the serum with a mixture of ascorbic acid and DMSO followed by filtration through a 0.45 μm cellulose acetate filter, the laborious precipitation with sodium sulfate can be avoided. The bilirubin fractions can then be separated on a large-pore (300 Å) reversed-phase column with a linear water–isopropanol gradient. First the bilirubin-diglucuronide is eluted, followed by bilirubin-monoglucuronide, *Bd* and *Bu* [44].

In another method for differentiation of bilirubin with HPLC, serum diluted with acetic acid is filtrated through a 0.45 μm filter, transferred to a polyacrylic-ester column and finally eluted with a linear pentasulfonic acid–acetonitrile gradient. Five bilirubin fractions can be detected in the eluate; the additional fifth fraction in comparison to the both methods described above is the (Z,E) or (E,Z) photoisomer of bilirubin. The elution sequence corresponds in principle to that of the Lauff method, with the exception that the photoisomer elutes between the bilirubin-monoglucuronide and *Bu* [45]. A disadvantage is the high cost of the procedure and the use of microfilters through which possible losses of *Bd* may arise. Additionally, the solvent acetonitrile is a carcinogenic substance [14].

HPLC permits the separation and simultaneous detection of different bilirubin fractions in serum. A disadvantage is the need for pre-treatment of the samples, with possible losses of bilirubin, which may not affect all fractions equally. With respect to the analysis of the HPLC it is problematic that the calibration is performed with *Bu*. The relative proportions of the individual fractions are then calculated on the assumption that the extinction coefficients of the various bilirubin fractions correspond to that of *Bu*. In fact, however, this is not known in detail. The measurement of the relative proportions of the fractions also carries the danger that errors in one fraction will recur in others, and in this way may accumulate. Irrespective of the method used, the procedures are too labour- and time-intensive to be practicable for routine analysis. The bilirubin taurate usually employed for quality control of the glucuronides is not suitable for this purpose because of irregular running times in the HPLC. Considering the instability of the glucuronides, there is no suitable control material. The HPLC procedures are therefore unsuitable as reference methods. At the moment their use is restricted purely to research investigations.

Measurement of direct bilirubin

The concepts of “direct” and “indirect” bilirubin are based on the behaviour of the various bilirubin fractions in the coupling reaction with diazonium salts. “Direct” bilirubin denotes that portion of the bilirubin that, without addition of an accelerator, reacts straightaway with the diazo-reagent. “Indirect” bilirubin, on the other hand, reacts rapidly only in the presence of an accelerator. The “direct” bilirubin measured with various procedures represents, however, only a more or less rough estimation of *Bc* or *Bc*+*Bd.* In virtually all tests *Bu* is detected to a varying extent as well while *Bc* and/or *Bd* are not quantitatively measured. Hence, it appears reasonable to replace the measurement of direct bilirubin as far as possible by the measurement of *Bc* [14, 16]. Under routine conditions, however, this is only possible with the multilayer-film technology (see above).

The procedures for measuring direct bilirubin are described below. Essentially, this involves modifications of the methods described above for the measurement of total bilirubin. However, the reaction conditions are varied in that way, that only certain bilirubin fractions react.

Diazo method

Procedures for measuring total bilirubin that are based on the coupling of bilirubin with diazonium salts are performed without addition of an accelerator. By definition, the bilirubin concentrations measured that way correspond to direct bilirubin [15, 16].

Since the reactivity of the *Bu* in the direct diazo reaction decreases with decreasing pH in the reaction mixture [14], a limited specificity for the measurement of *Bc* can be achieved through lowering the pH. Typically, first HCl and after an incubation time of five minutes the diazonium salt is added. An accelerator is not added. After a variable coupling time of about ten minutes the absorption of the azopigment can be determined at 540 nm or, after addition of alkaline tartrate, at 598 nm [14, 16]. Through pre-incubation in an acid medium the conversion of unconjugated bilirubin in the direct diazo reaction is minimized, which is achieved at the expense of a slight reduction in the reactivity of the directly reacting bilirubin fractions. From a clinical point of view, a slightly falsely too low measured direct bilirubin concentration, which in fact is attributable to *Bc*, seems to be more acceptable than false-positive measurement of a portion of *Bu* in the

direct diazo-reaction, that may be erroneously interpreted as conjugated hyperbilirubinaemia [14].

The combination of the absence of an accelerator and a low pH value of 4.75 modifies the bilirubin–azo coupling in such a way that *Bu* will not react and *Bd* will scarcely do so [14, 16]. Although with the described optimized reaction conditions the measured value for direct bilirubin approaches the concentration of *Bc*, it should explicitly be stated that the *Bc* concentration cannot be definitively measured by this procedure. In addition, the optimized method cannot be automated because of the long incubation time.

Enzymatic methods

These methods use the property of BOX to catalyse the oxidation of *Bc* and the greater part of *Bd* to biliverdin at low pH values between 3.7 and 4.5, while *Bu* is not converted. Hence, in an acid medium the BOX method measures only the direct bilirubin. Note that the wavelength for measurement must be changed to 460 nm, since under the chosen conditions the difference in absorption between bilirubin and a blank reaches a maximum at this wavelength range [14, 15, 46]. The various procedures differ mainly in respect of the buffer that is added. For example, the use of a phosphate buffer necessitates addition of urea in order to prevent precipitation of globulins, whereas use of a lactate–citrate buffer system can avoid this problem [14]. The concentrations of direct bilirubin determined by enzymatic procedures are lower than those from multilayer-film techniques, presumably because BOX will not completely convert the *Bd* [14] under the above-mentioned conditions. Further, a comparison of enzymatic procedures and diazo methods, particularly in the presence of *Bc* in the sample, reveals partially divergent results for direct bilirubin. This seems to be attributable to incomplete oxidation products of Bc, which arise in the acid medium during the performance of the BOX method [47].

It has been reported that at a pH of 10 only *Bc*, but not *Bu* or *Bd*, is oxidized to biliverdin by the BOX. Hence, a selective determination of the *Bc* concentration in the sample material would be achieved, and this concentration would be directly proportional to the decrease in absorption at 460 nm [14, 16]. But the specificity of the measurement is questionable: There is evidence for the oxidation of a portion of *Bu* by the BOX even at pH 10 [14].

Quality assurance

Preanalytics

Direct sunlight can lead to bilirubin losses of up to 50% within one hour, particularly if the sample is contained in a glass capillary tube. Even bright laboratory light can cause losses, with conjugated bilirubin appearing to be more susceptible than unconjugated or delta bilirubin. In order to avoid problems with haemolysis, the sample should be centrifuged within four hours after blood withdrawal, and the serum/plasma be separated. Protected from light and in closed vessels the bilirubin in serum or plasma is stable for ~3 days at room temperature or ~8 days in the refrigerator [48, 49].

Analysis

The new "Guidelines of the Federal Medical Association for quality assurance in laboratory medicine investigations" (RiLiBÄK) that come into force in 2008 specify that, for total bilirubin, two control samples are to be measured within 24 h. Over the concentration range 2–30 mg/dL the results should not deviate more than 13% from the target value of the control sample. Additionally, at the end of the control cycle the "quadratic mean deviation" of the measurement has to be calculated, for which likewise a maximum deviation of 13% is demanded. For controls in the measurement of neonate bilirubin, it is advisable to use special control sera with high bilirubin concentrations, e.g., Precibil (Roche, lyophilized) or Liquicheck Pediatric (Bio-Rad, fluid). The evaluation of other measurements of bilirubins, e.g., of direct bilirubin, is carried out with intralaboratory margins of error or the ranges that the supplier of the control serum has specified.

External quality assurance is – as before – compulsory four times each year and concerns only total bilirubin. The results of round-robin tests must not deviate by more than 22% from the target value of the control sample. However, the German reference institutions (Reference Institute for Bioanalysis of the DGKL and INSTAND e.V.) offer in addition to the round-robin tests for total bilirubin also specific round-robin tests, e.g., for direct bilirubin, unconjugated and conjugated bilirubin, and neonate bilirubin. By participating in these round-robin tests the quality control of the above-mentioned parameters can be reasonably supplemented.

Bilirubin in the amniotic fluid

Liley [50] proposed the measurement of so-called bilirubinoids in the amniotic fluid in order to estimate the degree of fetal compromise when haemolytic anaemia is suspected; however, this is now regarded as obsolete. Today the situation can better be characterized through noninvasive measurement of the fetal blood flow in the central artery of the umbilical cord by Doppler sonography.

Measurement of bilirubin in neonates

For lack of space the special problems that can arise in the measurement of bilirubin in neonates are not dis-

cussed here. See the literature regarding this diagnostically important issue [51].

Bilirubin assays on large-scale apparatus

Abbott, Beckman Coulter, Siemens and Roche are the four largest suppliers of laboratory diagnostics in Germany and manufacture large-scale clinical-chemical apparatus that enable bilirubin measurement in the field of routine clinical diagnostics.

For the measurement of total bilirubin, Beckman Coulter and Siemens use diazotized sulfanilic acid, which is produced from sulfanilic acid and sodium nitrite in the presence of HCl in ready-made reagent kits. In the system provided by Abbott, the diazo-component is synthesized from 2,4-dichloraniline and sodium nitrite, again in a medium acidified by addition of HCl. Roche markets two different procedures for measuring total bilirubin, depending on the apparatus used. The cobas kits use a 2,5-dichlorphenyl-diazonium salt according to the DPD method. In contrast, the modular system uses a new type of diazonium ion, 2-methyl-3-nitro-anilin diazonium, which is characterized by lower interference with haemoglobin and indican. As accelerators, Beckman Coulter adds caffeine, benzoate and acetate to the reaction mixture, and Siemens additionally to these three components also adds EDTA. On the other hand, Roche and Abbott use detergents to release the *Bu* from albumin. The photometric quantification of total bilirubin is achieved at various principal measurement wavelengths between 520 and 570 nm, differing from manufacturer to manufacturer.

For measurement of direct bilirubin, Beckman Coulter, Roche and Siemens use diazotized sulfanilic acid as a product of sulfanilic acid and sodium nitrite in an acidic medium (HCl). In contrast to this, the Abbott kit again uses the previously described diazonium ion on the basis of 2,4-dichloraniline. At the systems of Siemens and Abbott the principal measurement wavelengths are the same as those for the measurement of total bilirubin. In the case of direct bilirubin, the absorption is measured with the Beckman Coulter system at 560 nm instead of 520 nm, and with the Roche system irrespective of the apparatus at 546 nm, the principal measurement wavelength of total bilirubin on the modular system.

In addition it should be mentioned that, both for the measurement of total bilirubin and also for that of direct bilirubin, Abbott additionally offers a procedure in which bilirubin is oxidized by sodium nitrite to biliverdin. The conversion of bilirubin causes a decrease in the absorption at 444 nm; this decrease is, depending on the reaction conditions, proportional to the respective bilirubin fraction.

None of the manufacturers offers enzymatic methods or special manual methods.

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