A multi-component spectrophotometric method for simultaneous determination of total bilirubin, oxyhemoglobin, and methemalbumin in human sera

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ARTICLE INFO

ABSTRACT

A new multi-component spectrophotometric method was developed experimentally and theoretically to determine the accurate serum concentrations of the total bilirubin (TB), oxyhemoglobin (HbO$_2$), and methemalbumin (Mha) in healthy human adults and neonates with hemolytic jaundice. With respect to the experimental technique, the method of preparation of serum solution has been developed, like the use of distilled water as a solvent and centrifugation of serum solutions to clear the sample turbidity. The results of TB were compared to the diazo-assay. Theoretically, the formulas used for the calculation of the major components (TB, HbO$_2$, and Mha) in human sera have been derived based on the theory of multi-component spectrophotometric analysis and the mathematical Gaussian elimination method for matrix calculation. The method of multi-component spectrophotometry, suggested in this study for determination of TB, showed % error (3.1%–4.9%), indicating the high accuracy of the method. The small coefficients of variation (CV = 3.65%–5.1%) indicate the high precision of the method. The results showed higher values of serum TB ($p < 0.00005$), HbO$_2$ ($p < 0.001$), and Mha concentrations ($p < 0.00005$) in neonates, when compared to adults. The method is highly sensitive and accurate. It is inexpensive, precise, reproducible, and has the advantages of simplicity, speed, and can be computerized.

1. INTRODUCTION

Bilirubin is a breakdown product of hemoglobin heme. It is transported from the spleen to the liver and excreted into the bile. Bilirubin consists of an open chain of four pyrrole-like rings (tetrapyrrole). In heme, these four rings are connected into a larger ring, called a porphyrin ring. Free heme can be toxic, so nature evolved a family of microsomal heme oxygenase enzymes to degrade heme [1] and their blockade leads to greatly increased excretion of unmetabolized heme in the bile [2]. These enzymes cleave the heme ring to form biliverdin, iron, and carbon monoxide (CO). CO is excreted via the lungs and iron is utilized. Biliverdin is a green-colored pigment which is subsequently reduced to

bilirubin by the reduced form of nicotinamide adenine dinucleotide phosphate dependent cytosolic enzyme biliverdin reductase [3].

Bilirubin formed in peripheral tissues is transported to the liver by binding noncovalently to plasma albumin. In the liver, bilirubin is conjugated with glucuronic acid by the enzyme glucuronyltransferase, making it soluble in water: the conjugated version is also called "direct" bilirubin. Much of it goes into the bile and thus out into the small intestine.

Determination of total and direct bilirubin is important for the diagnosis of many diseases associated with hyperbilirubinemia. Hyperbilirubinemia results from the increase of bilirubin concentrations in blood plasma. Clinically hyperbilirubinemia appears as jaundice or icterus. Jaundice can usually be detected when the serum bilirubin level exceeds 2.0–2.5 mg/dl. When the level of bilirubin is between 1 and 2 mg/dl, it is known as latent jaundice [4]. Causes of hyperbilirubinemia: Total bilirubin:

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Increased hemolysis, genetic errors, neonatal jaundice, ineffective erythropoiesis, and hepatocellular disease (viral or drug-induced hepatitis, cirrhosis) [4,5].

Due to the various diseases associated with significant elevation of bilirubin, it is necessary for the recent studies to suggest new innovated diagnostic methods to facilitate diagnosis of these diseases. Several methods have been used to measure the total bilirubin in human blood sera. In some methods, bilirubin is converted to colored azobilirubin by diazotized sulfanilic acid and measured photometrically [6–8]. The use of many chemical reagents, like sulfanilic acid, hydrochloric acid, sodium nitrate, caffeine, sodium benzoate, tartrate, and NaOH, makes this method expensive. Furthermore, this method is laborious, time-consuming, and sensitive to hemolysis, which represents the main drawbacks of this method. Other enzymatic method depends on the reactivity of the enzyme "bilirubin oxidase" to bilirubin at pH 8 in the presence of SDS for determination of total bilirubin [9]. The same enzyme was used previously for the determination of plasma hemoglobin in the presence of bilirubin [10]. The use of costly enzyme and chemicals make these methods expensive.

Other methods depend on direct spectrophotometry of serum solution at two wavelengths [11–13]. These methods are expensive since they depend on the use of expensive caffeine reagent for sample preparation. In addition, no centrifugation of serum solutions was performed, which makes these methods sensitive to turbidity. Lipids-induced turbidity of serum samples, as in lipemia, interfered positively with these methods [12]. Furthermore, these methods were not applied for the determination of HbO_2 concentration in human serum.

Recently, a direct spectrophotometric method [14] with a bilirubinometer (LEICA UNISTAT, Leica I) was used to determine the TB concentrations in plasma of newborns. However, this method is characterized by a high imprecision (CV), which according to the manufacturer is 15% and 10% at bilirubin concentrations of 21 μmol/L (1.2 mg/dl) and 130 μmol/L (7.6 mg/dl), respectively. Moreover, the effects of lipemia as a source of turbidity of plasma samples were not evaluated and taken into account in this method. Since no centrifugation at high speed was performed, it is expected that the turbidity induced by plasma lipids is a source of error and may be the cause of the high imprecision of this method. In addition, this method was not applied for the determination of HbO_2 concentration in human serum.

All direct spectrophotometric methods, used for determination of bilirubin in human serum, are based on absorbance measurement at two wavelengths to account for the absorption of HbO_2. Another component which has a significant absorption in the wavelength range of absorption of bilirubin is the Mha [15]. The presence of this component in blood plasma leads to an error in bilirubin values if its absorption is not taken into account during the calculation [12]. Therefore, the two-wavelength method yields incorrect values of bilirubin. To solve this problem, we have taken into account the absorption contribution of Mha, as a third component beside HbO_2 during the calculation of bilirubin concentration in human serum. Therefore, we should use a three-wavelength method to account for this absorption, by using the technique of multi-component spectrophotometric analysis suggested in this article. Since no correction for Mha was performed in previous two-wavelength direct spectrophotometric methods [11–14], it is expected that these methods yield incorrect values of total bilirubin. Mha has absorbance maxima that could interfere with these spectrophotometric methods and a significant positive interference of this component with these methods was noted in a previous study [12]. Moreover, all these direct spectrophotometric methods have not been applied for the determination of Mha in human sera.

Another serum pigment, which has lower molar absorptivity in the range of bilirubin absorption, and may interfere, is transferrin [15]. Since no correction for this component was performed in previous direct two-wavelengths spectrophotometric methods [11–14], it is expected that these methods yield incorrect values of total bilirubin.

Other pigments, such as carotenoids, also absorb at 460 nm, the absorption maximum of bilirubin, thus limiting the two-wavelength direct spectrophotometric assay to neonates < 2–3 weeks of age [14], whose a low mean mass concentration of carotenoids in serum of 0.17 mg/l (0.32 μmol/l) [15]. In direct spectrophotometry of serum from neonates, the influence of carotenoids was negligible [11]. However, the influence of carotenoids cannot be neglected in adults and may interfere with these direct spectrophotometric methods, which show positive interference from carotene [12]. Therefore, correction for the absorption of this pigment is necessary for accurate determination of bilirubin in adults, by using these methods.

Therefore, this practical study aimed to develop a new direct multi-component spectrophotometric method for accurate and simultaneous determination of total bilirubin, HbO_2, and Mha in human adults and neonates sera in order to overcome the drawbacks of previous methods.

2. MATERIALS AND METHODS

2.1. Subjects

Twenty healthy adult volunteers were investigated in this study. The volunteers had to fulfill the following criteria: not suffering from any hemolytic anemia, hepatitis, or liver failure. Another group of four human neonates with hemolytic jaundice were also investigated in this study. All subjects contributing to this study were admitted to the medical research center of excellence at the national research center. The written consent of the volunteers was obtained. The study was approved by the Research Ethics Committee of the National Research Centre.

2.2. Blood collection

Blood samples were withdrawn from humans by venipuncture and collected into plastic tubes without anticoagulant. Then, blood samples were allowed to coagulate for 2 minutes at room temperature. This is important because the long period of coagulation was found to be accompanied by high hemolysis and turbidity. The blood samples were centrifuged at 4,000 rpm for 15 minutes to separate the serum. Immediately after separation, serum samples were centrifuged at 6,000 rpm for 10 minutes to eliminate any erythrocytes or lipids that may have been present. Clear sera were analyzed immediately.

2.3. Determination of total bilirubin, oxyhemoglobin, and methemalbumin

The levels of the total bilirubin, HbO_2, and Mha in human serum were determined by the following multi-component spectrophotometric method.

2.3.1. Materials and sample preparation

For absorbance measurements, 100 µl of the human serum is added to 1 ml of ice-cold distilled water (pH 9.3). After mixing and incubation in a refrigerator at 4°C for 10 min, centrifuge the serum solution at 10,000 rpm and 4°C for 10 minutes to clear the sample turbidity. Separate the purified serum solution for absorbance measurements, by pouring the clear supernatant into another clean tube.

2.3.2. Measurements and calculations

The absorbance measurements for the purified adult human serum solution were made at four wavelengths (λ = 469.8, 576, 600, and 700 nm), using a Cary UV/VIS double-beam spectrophotometer (model 100 UV-VIS), manufactured by Agilent Technologies, Australia. The absorbance measurements for the purified neonate's serum solution were made at four wavelengths (λ = 466, 576, 600, and 700 nm). The spectrophotometer was adjusted at a spectral bandwidth of 2.0 nm and a quartz cuvette of 1.0 cm light path was used for absorbance measurement. A cuvette filled with distilled water was used as a blank. The absorbances of the blank were measured first using a cleaned quartz cuvette against the air as a reference. Then the absorbances of the human serum solution were measured against the air as a reference using the same blank cuvette without any further washing or cleaning. The absorbances $A_{466}/A_{469.8}$, A_{576}, A_{600}, and A_{700} of the serum pigments in solution were calculated by subtracting the absorbances of the blank from the absorbances of the solution measured at the same wavelengths.

The three absorbances $A_{469.8}$, A_{576}, and A_{600} should be corrected for the very low residual turbidity remaining after centrifugation of serum solution, by subtracting from the absorbance at 700 nm (A_{700}), where the serum pigments have negligible absorption, the absorption at this wavelength is mainly due to serum turbidity. The three absorbances should also be corrected for transferrin and carotenoids (in the form of β-carotene) present in human serum, by using the usual reference concentrations and molar absorptivity of these components in human serum [15], as the following:

$$A'_{469.8} = A_{469.8} - A_{700} - 0.0168338 \tag{1}$$

$$A'_{576} = A_{576} - A_{700} - 0.00286364 \tag{2}$$

$$A'_{600} = A_{600} - A_{700} - 0.00109091 \tag{3}$$

where $A'_{469.8}$, A'_{576}, and A'_{600} are the corrected absorbances of serum solution, and 0.0168338, 0.00286364, and 0.00109091 are the correction factors for transferrin and carotenoids at 469.8, 576, and 600 nm, respectively. Since in practice, hypercarotinemia is rare [12], we used the usual reference value of β-carotene in adult serum (0.53 µmol/l) [15] to correct for the three absorbances used in our method.

For human adults, the 9 millimolar absorptivities of TB, HbO_2, and Mha, determined previously [15], were substituted into three linear equations of the type described by the theory of multi-component spectrophotometric analysis [16], with the three unknown concentrations of serum pigments (C_{TB}, C_{HbO2}, and C_{Mha}).

$$A'_{469.8} = 47.7C_{TB} + 8.1C_{HbO2} + 8.3C_{Mha} \tag{4}$$

$$A'_{576} = 0.07C_{TB} + 14.5C_{HbO2} + 4.95C_{Mha} \tag{5}$$

$$A'_{600} = 0.00C_{TB} + 0.8C_{HbO2} + 4.95C_{Mha} \tag{6}$$

where the absorption bands at wavelengths 469.8, 576, and 600 nm represent the near-maximum absorptions of bilirubins, HbO_2, and Mha, respectively, and these wavelengths are the isobestic points of both unconjugated and conjugated bilirubins in adult serum [15,17]. At these isobestic points, the conjugated and unconjugated bilirubins have the same molar absorptivity and, therefore, both components can be represented as a single one (i.e., total bilirubin), which can be calculated by absorbance measurement at three wavelengths instead of four, taking in account the absorption contribution of the other two major components (HbO_2 and Mha). The above linear system of equations can be represented in the matrix form as:

$$\begin{bmatrix} 47.7 & 8.1 & 8.3 \\ 0.07 & 14.5 & 4.95 \\ 0.0 & 0.8 & 4.95 \end{bmatrix} \times \begin{bmatrix} C_{TB} \\ C_{HbO2} \\ C_{Mha} \end{bmatrix} = \begin{bmatrix} A'_{469.8} \\ A'_{576} \\ A'_{600} \end{bmatrix}$$

This linear system of equations was solved by mathematical manipulation, using the Gaussian elimination method for matrix calculation [18], to yield the following formulas of adults serum pigments:

$$C_{Mha} = \frac{A'_{600} - 0.0552176 A'_{576} + 8.10322234 \times 10^{-5} A'_{469.8}}{4.677345} \tag{7}$$

$$C_{HbO2} = \frac{A'_{576} - 1.4675052 \times 10^{-3} A'_{469.8} - 4.9378197 C_{Mha}}{14.488113} \tag{8}$$

$$C_{TB} = \frac{A'_{469.8} - 8.1C_{HbO2} - 8.3C_{Mha}}{47.7} \tag{9}$$

where $A'_{469.8}$, A'_{576}, and A'_{600} are the corrected absorbances measured experimentally at wavelengths 469.8, 576, and 600 nm, respectively, for purified, diluted, and aqueous serum solution, and C_{TB}, C_{HbO2}, and C_{Mha} calculated by these equations represent the concentrations (in mmol/l) of various pigments in this diluted serum solution.

For neonates, the three absorbances A_{466}, A_{576}, and A_{600} should be corrected for the very low residual turbidity remaining after centrifugation of serum solution, by subtracting from the absorbance at 700 nm (A_{700}). The three absorbances should be corrected also for transferrin and carotenoids (in the form of β-carotene) present in human serum, by using the usual reference

concentrations and molar absorptivity of these components in human serum [15], as the following:

$$A'_{466} = A_{466} - A_{700} - 0.0110495 \tag{10}$$

$$A'_{576} = A_{576} - A_{700} - 0.0021356 \tag{11}$$

$$A'_{600} = A_{600} - A_{700} - 0.00081356 \tag{12}$$

where A'_{466}, A'_{576}, and A'_{600} are the corrected absorbances of serum solution, and 0.0110495, 0.0021356, and 0.00081356 are the correction factors for transferrin and carotenoids at 466, 576, and 600 nm, respectively. Since in practice, hypercarotinemia is rare [12], we used the usual reference value of β-carotene in neonates serum (0.317 µmol/l) [15] to correct for the three absorbances used in our method.

For human neonates, the 9 millimolar absorptivities of TB, HbO$_2$, and Mha, determined previously [15], were substituted into three linear equations of the type described by the theory of multi-component spectrophotometric analysis [16], with the three unknown concentrations of serum pigments (C_{TB}, C_{HbO2}, and C_{Mha}).

$$A'_{466} = 50.4 C_{TB} + 8.8 C_{HbO2} + 8.49 C_{Mha} \tag{13}$$

$$A'_{576} = 0.09 C_{TB} + 14.5 C_{HbO2} + 4.95 C_{Mha} \tag{14}$$

$$A'_{600} = 0.00 C_{TB} + 0.8 C_{HbO2} + 4.95 C_{Mha} \tag{15}$$

where the absorption bands at wavelengths 466, 576, and 600 nm represent the near-maximum absorptions of bilirubins, HbO$_2$, and Mha, respectively, and these wavelengths are the isobestic points of both unconjugated and conjugated bilirubins in infant serum [15]. The above linear system of equations can be represented in the matrix form as follows:

$$\begin{bmatrix} 50.4 & 8.8 & 8.49 \\ 0.09 & 14.5 & 4.95 \\ 0.0 & 0.8 & 4.95 \end{bmatrix} \times \begin{bmatrix} C_{TB} \\ C_{HbO2} \\ C_{Mha} \end{bmatrix} = \begin{bmatrix} A'_{466} \\ A'_{576} \\ A'_{600} \end{bmatrix}$$

This linear system of equations was solved by mathematical manipulation, using the Gaussian elimination method for matrix calculation [18], to yield the following formulas of neonates serum pigments:

$$C_{Mha} = \frac{A'_{600} - 0.05523227148 A'_{576} + 8.86290559 \times 10^{-5} A'_{466}}{4.677437617} \tag{16}$$

$$C_{HbO2} = \frac{A'_{576} - 1.78571428 \times 10^{-3} A'_{466} - 4.934839286 C_{Mha}}{14.48428571} \tag{17}$$

$$C_{TB} = \frac{A'_{466} - 8.8 C_{HbO2} - 8.49 C_{Mha}}{50.4} \tag{18}$$

where A'_{466}, A'_{576}, and A'_{600} are the corrected absorbances measured experimentally at wavelengths 466, 576, and 600 nm, respectively, for purified, diluted, and aqueous serum solution, and C_{TB}, C_{HbO2}, and C_{Mha} calculated by these equations represent the concentrations (in mmol/l) of various pigments in this diluted serum solution.

The C_{TB}, Mha, and HbO$_2$ concentrations in human serum (in µmol/l) can be determined by using the following equations:

$$C'_{Mha} = 11 \times 10^3 C_{Mha} \text{ µmol / L} \tag{19}$$

$$C'_{HbO2} = 11 \times 10^3 C_{HbO2} \text{ µmol / L} \tag{20}$$

$$C'_{TB} = 11 \times 10^3 C_{TB} \text{ µmol / L} \tag{21}$$

where 11 is the dilution factor of human serum and 10^3 is the conversion factor for mmol/l to µmol/l. For conversion to the TB concentration in mg/dl, mg/dl × 17.1 = µmol/l [19].

2.4. Determination of total bilirubin by the diazo-method

Total bilirubin concentration was determined also by the diazo-assay, as a reference method, using the bilirubin kit (Diamond Diagnostics Company, Cairo, Egypt), according to the Doumas version of the Jendrassik-Grof method [6].

2.5. Data analysis

Data were presented as the mean ± standard deviation (SD) values. The Student's *t*-test was used for the determination of the level of significance of the difference between the values of total bilirubin determined by the two methods, using the statistical program (Statistics Calculator). The *t* distribution was used to calculate the 95% confidence interval of the parameter assessed.

The precision of the method was assessed by the CV values and the 95% confidence intervals of the parameter assessed for a serum sample from a single adult subject.

Linear regression analysis was used to determine the best-fit line, with the corresponding fit function, including the intercept and the slope, for the results of total bilirubin determined by our method versus those determined by the reference diazo-method. Pearson's correlation coefficient (*r*) was used to determine this relationship, assessing the strength of association between the two variables.

By using the advanced database (Clipper)-language, a computer program, called Atef's program, was designed, by which we can estimate easily the concentrations of TB, HbO$_2$, and Mha in human sera, based on the Equations (1)–(21) mathematically derived for human's serum-pigments. The software is characterized by simplicity, speed, and high accuracy.

3. RESULTS

3.1. Precision and accuracy

The results of reproducibility, precision, and accuracy of the multi-component spectrophotometric method, suggested in this article for determination of TB, are shown in Table 1. The within-run precision of the method was evaluated by measuring the serum concentrations of total bilirubin for eight replicates of serum from a single adult subject in a single run and then calculating the values of SD for these samples. While the run-to-run reproducibility and precision of the method were evaluated by measuring the serum concentrations of total bilirubin for 16 replicates of serum from a single adult subject in different runs and then calculating the values of SD for these samples.

Table 1: Repeatability, reproducibility, precision, and accuracy of the multi-component spectrophotometric method for the total bilirubin in a healthy adult human serum.

Parameter	Total bilirubin (µmol/l)(within-run determinations, $n = 8$) (the reference value = 20.52 µmol/l)[†]	Total bilirubin (mg/dl) (within-run determinations, $n = 8$)(the reference value = 1.2 mg/dl)[†]	Total bilirubin (µmol/l)(run-to-run determinations, $n = 16$) (the reference value = 20.52 µmol/l)[†]	Total bilirubin (mg/dl)(run-to-run determinations, $n = 16$) (the reference value = 1.2 mg/dl)[†]
Mean ± SD	19.873 ± 1.032	1.162 ± 0.06	19.506 ± 0.959	1.141 ± 0.056
Coefficient of variation (CV)	5.1%	5.1%	4.9%	4.9%
95% Confidence interval for mean	19.009–20.736	1.112–1.212	18.995–20.017	1.111–1.170
Range of the 95% confidence interval	1.727	0.1	1.022	0.059
% Error	−3.1%	−3.1%	−4.9%	−4.9%

[†]The values of total bilirubin measured by the diazo-reference method.
The % error of the values of total bilirubin measured by our method was calculated as the difference between the mean value of 8–16 determinations measured by this method and the reference actual value measured by the reference diazo-method, relative to the reference actual value.
n indicates the number of replicates.

The method provided reproducible results of TB at the rates of 5.1% and 4.9%, respectively, indicating the high reproducibility of this method. The small ranges of 95% confidence intervals indicate the high precision of the mean values, while the small CV indicate the high precision of individual values, determined by this method. The within-run precision of the method is relatively high (CV 5.1%) and between-run precision is also high (CV 4.9%) at TB concentration of 20.52 µmol/L (1.2mg/dl). While the reproducibility and precision of the method are higher (CV 3.65%) at TB concentration of 49.59 µmol/l (2.9 mg/dl). On the other hand, the small values of % error (3.1%–4.9%) indicate the high accuracy of the method.

3.2. Effects of hemolysis (HbO$_2$)

We investigated the effect of HbO$_2$ on the results of TB determined by the multi-component spectrophotometry. We verified this effect in the usual concentration range in healthy adult subjects from 0.139 µmol/l (0.002 g/l) to 15.611 µmol/l (0.251 g/l). The results of these observations, which are summarized in Table 2, show that the multi-component spectrophotometric method is insensitive to HbO$_2$ up to 15.611 µmol/l (0.251 g/l). Measurement of absorbance at three different wavelengths by direct multi-component spectrophotometry can be used to correct completely for the influence of HbO$_2$ up to 15.611 µmol/l (0.251 g/l).

3.3. Effects of turbidity

The three absorbances $A_{469.8}$, A_{576}, and A_{600} used in the study were corrected for the very low residual turbidity, remaining after centrifugation of serum solution, by subtracting from the absorbance at 700 nm (A_{700}), where the serum pigments have negligible absorption, the absorption at this wavelength is mainly due to serum turbidity. We investigated the effect of turbidity represented by the absorbance at 700 nm (A_{700}) in diluted serum samples on the results of TB determined by the multi-component spectrophotometry and also verified this effect in the usual A_{700}-range of a diluted aqueous solution of serum, from 0.0022 to 0.0203. The results of these observations, which are summarized in Table 3, show that the multi-component spectrophotometric method is insensitive to turbidity up to A_{700} of 0.0203. The correction of absorbances $A_{469.8}$, A_{576}, and A_{600} used in the study by subtracting from the absorbance of turbidity

Table 2: Effects of hemolysis (HbO$_2$ concentration) in serum samples on the total bilirubin determined by the multi-component spectrophotometry.

HbO$_2$ concentration (g/l)	HbO$_2$ concentration (µmol/l)	% of original bilirubin, i.e., those measured by multi-component spectrophotometry relative to that determined by the reference diazo-method[†]
0.002	0.139	96.83
0.023	1.431	95.08
0.105	6.544	104.13
0.150	9.327	99.88
0.201	12.529	106.48
0.251	15.611	102.61

[†]Original concentration of bilirubin was 20.52 µmol/l (1.2 mg/dl), as determined by the reference diazo-method, in sera from a healthy adult subject. The values are the means of 5–10 determinations in sera from a single healthy adult subject.

Table 3: Effects of turbidity represented by the absorbance at 700 nm in diluted serum samples on the total bilirubin determined by the multi-component spectrophotometry.

Absorbance at 700 nm[†]	% of original bilirubin, i.e., those measured by multi-component spectrophotometry relative to that determined by the reference diazo-method[‡]
0.0022	104.08
0.0045	97.47
0.0071	99.55
0.0118	100.64
0.0203	101.72

[†]Absorbance at 700 nm was measured in diluted aqueous solution of sera from a healthy adult subject at a dilution factor 11.
[‡]Original concentration of bilirubin was 20.52 µmol/l (1.2 mg/dl), as determined by the reference diazo-method, in sera from a healthy adult subject. The values are the means of 3–10 determinations in sera from a single healthy adult subject.

band (A_{700}), as given from equations (1–3), can be used to correct completely for the influence of residual turbidity up to A_{700} of 0.0203.

3.4. Comparison of the method with the reference diazo-method

The results of TB concentrations in blood sera of healthy adult subjects are illustrated in Table 4. The determined TB

Table 4: The total bilirubin concentrations in normal adult human sera as determined by the multi-component spectrophotometric method and diazo-reference method.

Specimen	Total bilirubin (µmol/l) by the diazo-reference method	Total bilirubin (mg/dl) by the diazo-reference method	Total bilirubin (µmol/l) by multi-component spectrophotometry	Total bilirubin (mg/dl) by multi-component spectrophotometry
1	10.26	0.6	10.245	0.599
2	13.68	0.8	12.011	0.702
3	20.52	1.2	19.873	1.162
4	8.55	0.5	6.909	0.404
5	10.26	0.6	10.073	0.589
6	8.55	0.5	6.860	0.40
7	9.576	0.56	9.217	0.539
8	10.26	0.60	9.555	0.559
9	7.695	0.45	7.832	0.458
10	8.55	0.5	7.815	0.457
11	12.825	0.75	12.673	0.740
12	6.84	0.4	7.175	0.419
Range	(6.84–20.52)	(0.4–1.2)	(6.86–19.873)	(0.4–1.162)
Mean ± SD	10.630 ± 3.685	0.621 ± 0.215	10.020 ± 3.648	0.586 ± 0.213
p-value			0.05*	0.05*
Mean of absolute values of total error			0.689	0.040
Mean of absolute values of relative error in %***			6.94%	6.95%

*Indicates the p-value of the difference between the total bilirubin concentrations measured by the multi-component spectrophotometric method and the diazo-reference method.

***The relative error of the values of total bilirubin measured by our method was calculated as the difference between the values measured by our method and the reference diazo-method, relative to the value measured by the reference method.

Values of total bilirubin measured by our method are means of duplicate determinations.

concentrations ranged between 0.4 mg/dl and 1.2 mg/dl (6.84–20.52 µmol/l) with a mean value of 0.621 ± 0.215mg/dl, using the reference diazo-assay. While those determined by the multi-component method were 0.4–1.162 mg/dl (6.86–19.873 µmol/l) with the mean value of 0.586 ± 0.213mg/dl. The very close agreement between individual total bilirubin values determined by both methods is demonstrated by statistical analysis with the t-test for paired experiments. The multi-component spectrophotometry, using distilled water as a solvent, showed the statistically insignificant difference, using a significance level $p = 0.05$, in comparison with the reference method, indicating the validity of the method to determine the total bilirubin concentration, by using only the distilled water as a solvent.

The values of TB assessed by the method are generally close to the reference actual values determined by the reference diazo-method. The mean total error for total bilirubin estimated by the method is about 0.04 mg/dl of mean relative value 6.95%. The small relative errors of the values measured by our method indicate the high accuracy of this method. Another evidence for this high accuracy comes from the correlation study and linear regression analysis. Figure 1 shows a linear regression plot for serum samples from adult subjects. The results show a good correlation with the reference diazo-method: a slope of 0.981, an intercept of −0.493, and a correlation coefficient of 0.998.

3.5. HbO$_2$ and Mha concentrations in healthy adults

The results of HbO$_2$ and Mha concentrations in blood sera of healthy adult subjects are illustrated in Table 5. The determined normal HbO$_2$ concentrations ranged between 0.0026 and 0.0516 g/l (0.162–3.20 µmol/l) with a mean value of 0.0333 ± 0.0164 g/l (2.069 ± 1.024 µmol/l). Reproducibility (CV) of the method is within ±4.6% and 6.1% at HbO$_2$ concentrations 1.431 and 6.544 µmol//l, respectively. The determined normal Mha concentrations ranged between 2.252 and 8.026 µmol/l with a mean value of 5.983 ± 1.833 µmol/l. Reproducibility (CV) of the method is within ±7.5% at Mha concentration 8.026 µmol/l.

3.6. TB, HbO$_2$, and Mha concentrations in neonates

The mean ± SD values of TB, HbO$_2$, and Mha concentrations in normal adult and neonates human sera, as determined by the multi-component spectrophotometric method, are illustrated in Table 6. The results showed higher basal values of serum TB (<0.00005), HbO$_2$ ($p < 0.001$), and Mha concentrations ($p < 0.00005$) in neonates when compared to adults.

4. DISCUSSION

During the current study, a new method based on principles of the multi-component spectrophotometric analysis was developed and found to be suitable for the estimation of TB, HbO$_2$, and Mha concentrations in adult human sera. This developed method requires taking into account all absorption contributions of all main components in blood sera, like bilirubin, HbO$_2$, and Mha. An advantage of this new method is its independence of the sample hemolysis because it allows the correction for HbO$_2$ by measurement at 469.8 and 576 nm, as compared to previous diazo-methods [6–8], which are sensitive to hemolysis [11].

$$y = 0.981x - 0.4928$$
$$r = 0.998$$
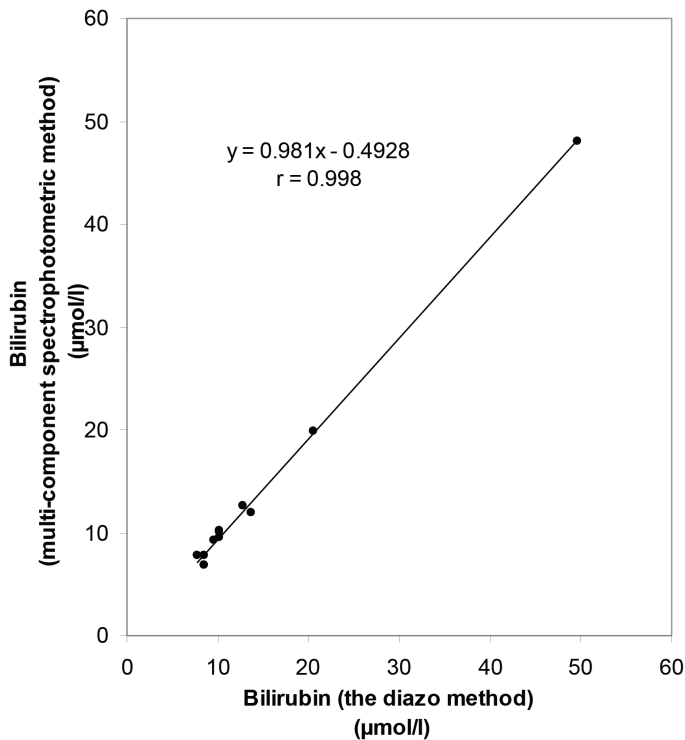
Figure 1: Comparison of the multi-component spectrophotometric method and the diazo-reference method used for determination of total bilirubin in adult human sera. Samples from healthy adults and an adult with jaundice were analyzed by both the multi-component spectrophotometric and diazo-reference methods. Linear regression analysis was used to determine the best fit line, with the corresponding fit function, with a slope of 0.981, an intercept of -0.493, and a correlation coefficient of 0.998.

Table 5: The oxyhemoglobin (HbO_2) and methemalbumin (Mha) concentrations in normal adult human sera, using the multi-component spectrophotometric method.

Specimen	HbO_2 (μmol/l)	HbO_2 (g/l)	Mha (μmol/l)
1	1.140	0.0184	6.502
2	1.334	0.0215	8.026
3	2.690	0.0430	2.252
4	0.162	0.0026	3.675
5	0.934	0.0150	6.758
6	1.352	0.0218	5.579
7	3.206	0.0516	7.279
8	3.059	0.0493	7.895
9	2.607	0.0419	4.214
10	2.783	0.0448	5.508
11	2.453	0.0395	6.178
12	3.116	0.0502	7.934
Range	(0.162–3.206)	(0.0026–0.0516)	(2.252–8.026)
Mean ± SD	2.069 ± 1.024	0.0333 ± 0.0164	5.983 ± 1.833

Values are means of duplicate determinations.

The absorbance measurement of human serum solution at 576 nm, where bilirubin has a minimum absorption and HbO_2 has a maximum absorption [15], allows the determination of HbO_2 concentration in the presence of bilirubin in the collected blood sera, by using our simple method, instead of using the costly enzyme "bilirubin oxidase" in a previous study [10]. The absorbance measurement of serum solution at the three wavelengths, 469.8, 576, and 600 nm, allows the corrections for bilirubin and Mha during the calculation of HbO_2 concentration [see the Equation (8)]. Therefore, the method yields more correct values of HbO_2 in human serum, as compared to other direct spectrophotometric methods used for HbO_2 determination [20,21], which are sensitive to high levels of bilirubin and Mha. Our method yields values of HbO_2 up to 0.0516 g/l in normal human serum, which are in agreement with a previous study [22], which reported values of free hemoglobin up to 0.05 g/l in normal human serum, using 4-aminophenazone as the chromogen. Reproducibility (CV) of the method is within ±4.6% and 6.1% at HbO_2 concentrations 1.431 and 6.544 μmol//l, which is significantly higher than that of the previous direct spectrophotometric method [23], which showed poor reproducibility within ±25%.

Another advantage of the method is that it allows the correction for Mha, by measurement of the absorbances at 600 nm, the near-maximum absorption band of Mha, in addition to the other bands at 469.8 and 576 nm [see the Equation (9)]. Therefore, it yields more correct values of TB in human sera, as compared to previous two-wavelength direct spectrophotometric methods, which showed positive interference of Mha [12].

Moreover, the absorption measurement at the isobestic points (469.8, 576, and 600 nm) of unconjugated and conjugated bilirubins allows accurate determination of total bilirubin as the sum of the two components (UB and CB) for human adult sera, which contain a significant proportion of CB, as compared to other direct spectrophotometric methods [14,15]. As the disadvantage of other direct spectrophotometric methods, TB is false overestimated in plasma containing "directly-reacting" pigment than in specimens not containing bilirubin in this form [24]. Two-wavelength formulas, used in other direct spectrophotometric methods, have yielded correct test results for infant sera with very low content of CB [15]. The use of the same formulas to estimate the total bilirubin in adult sera, with a high content of CB, yields false increased incorrect values.

Another advantage of the method is that it is inexpensive because it does not need expensive chemicals, like the caffeine reagent used in the previous direct spectrophotometric methods [11–13]. It has been reported that caffeine reagent clears the turbidity of human sera [11]. Since in this method we performed the centrifugation at high speed to clear this turbidity, we do not need the caffeine reagent, which represents an advantage of our method from the economic point of view.

Furthermore, the method allows the determination of total bilirubin with high accuracy, sensitivity, and reproducibility, by using only distilled water as a solvent. Moreover, it yields results of TB, which are in agreement with those determined by the reference diazo-method, confirming the validity of our assay to determine the TB, by using only distilled water as a solvent, which represents an advantage of the new method from the economic point of view, as compared to previous methods [6–9]. Furthermore, the method allows measurement of TB by using the

Table 6: The total bilirubin (TB), oxyhemoglobin (HbO$_2$), and methemalbumin (Mha) concentrations in sera of normal human adults and neonates with jaundice, using the multi-component spectrophotometric method.

Parameters	Adults (*n* = 12)	Neonates (*n* =4)	*p*-value
TB (μmol/l)	10.020±3.648(6.86–19.873)	174.582 ± 95.855(83.003–284.093)	<0.00005
HbO$_2$ (μmol/l)	2.069±1.024(0.162–3.206)	57.965± 48.805(25.436–130.365)	<0.001
Mha (μmol/l)	5.983±1.833(2.252–8.026)	51.493± 28.929(13.951–83.759)	<0.00005

The values expressed as mean ± S.D. *n* is the number of the individuals in each group; the values between parentheses represent the ranges of various parameters. *p* indicates the two-tailed probability of the significance of differences between the means of the two groups.

conventional spectrophotometer rather than the expensive LEICA bilirubinometer used in a previous study [14], which represents an advantage of this method from the economic point of view.

The results of this method showed values of TB up to 1.162 mg/dl in normal adult human serum, by using distilled water as a solvent. In agreement with these results, previous studies [4,25] reported values of TB up to 1.2 mg/dl in normal adult human sera, confirming the validity of the method. It yields values with a mean total error for a total bilirubin of about 0.04 mg/dl of mean relative value 6.95%. The allowable total error for total bilirubin is 0.4 mg/dl or 20% according to CLIA criteria [26]. These results indicate the high accuracy of the method. The results showed a good correlation with the reference diazo-method [6]: $r = 0.998$, indicating the high accuracy of the method.

In addition, the centrifugation of serum solutions at 10,000 rpm allows clearance of the sample turbidity induced by plasma lipids, in contrast to the previous direct spectrophotometric methods [11–14], in which no centrifugation was performed. Lipids-induced turbidity of serum samples, as in lipemia, interfered positively with these methods [12].

This method is characterized by the high sensitivity because it was able to detect TB, HbO$_2$, and Mha concentrations as low as 6.86, 0.162, and 2.252 μmol/l, respectively. Moreover, our method is precise as indicated by the small CV (3.6%–5.1%), in contrast to the previous direct spectrophotometric method [14], which showed a high imprecision (CV) of 15% at a bilirubin concentration of 21 μmol/l (1.2 mg/dl).

In addition to TB, this method allows determination of Mha, by using distilled water as a solvent, instead of expensive chemicals used in previous analytical methods [27–31]. This method yields more correct values of Mha since it allows the corrections for bilirubin and hemoglobin in the serum sample, by measurement of the absorbances at 469.8 and 576 nm, which represent the near-maximum absorptions of bilirubin and HbO$_2$, respectively [see the Equation (7)]. Therefore, this method for Mha determination is not affected by the presence of bilirubin and hemoglobin, as compared to the previous second-derivative spectrophotometric method [31], which is sensitive to these components. This method yields values of Mha up to 8.026 μmol/l in normal human serum, which are in agreement with a previous study [27], which reported values of Mha up to 9.2 μmol/l (0.6 mg of hematin/100 ml) in normal human plasma, using another method. Reproducibility (CV) of the method is within ±7.5%, which is consistent with that of a previous method [28].

The level of plasma Mha is elevated after severe intravascular hemolysis and in some patients with hemorrhagic pancreatitis. It is formed by the breakdown of hemoglobin to hematin which combines with albumin to give Mha. Determination of Mha by this method is important for the diagnosis of patients with intravascular hemolysis and hemorrhagic pancreatitis who showed an elevation in this pigment concentration in their plasma [28,32,33]. As shown in the present study, Mha concentration is significantly higher in neonates with hemolytic jaundice as compared to healthy adults.

Moreover, this method allows accurate determination of serum HbO$_2$ concentration, which increases in cases of intravascular hemolysis [20,34] and therefore has the importance of diagnosis of this disease. As shown in the present study, HbO$_2$ concentration is significantly higher in neonates with hemolytic jaundice as compared to healthy adults.

5. CONCLUSIONS

In conclusion, the multi-component spectrophotometric method, suggested in this study for determination of TB, HbO$_2$, and Mha in human sera, is highly reproducible and precise. The method is rapid, highly accurate, and sensitive. Furthermore, the method is inexpensive since it is based on using distilled water as a solvent and conventional spectrophotometer. In addition, the method is simple and can be computerized. Moreover, the method can be used, simultaneously, to determine concentrations of the TB, HbO$_2$, and Mha concentrations in human sera. Our method can be used to diagnose neonates with hemolytic jaundice characterized by high levels of TB, HbO$_2$, and Mha.

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